REMARKS

Claims 1, 10, 11, 15, 25, 29-32 and 35-65 are pending in this application. By this Amendment, claims 1, 10, 15 and 31 are amended, and claims 35-65 are added.

No new matter is added by this Amendment. Support for the language added to the claims can be found throughout the specification. For example, support for the language added to claim 1 can be found on page 2, lines 16-17 and page 16, lines 17-19 of the original specification. Support the new claims can be found throughout the claims and the original specification, for example at page 2, lines 14-18, lines 32-34 and page 3, lines 3-5.

The courtesies extended to Applicants' representatives by Examiner Baskar at the personal interview held December 8, 2006, are appreciated. The reasons presented at the interview as warranting favorable action are incorporated into the remarks below and constitute Applicants' record of the interview.

I. Rejection Under 35 U.S.C. §112, first paragraph

A. Written Description

Claims 1, 10, 11, 15, 25 and 29-32 were rejected under 35 U.S.C. §112, first paragraph, as allegedly being unsupported by an adequate written description in the specification. This rejection is respectfully traversed.

The Patent Office alleges that only one strain of *Tropheryma whippelii* has been cultured, and that given that different strains exist, the structure and function of other unknown strains as claimed are not supported by the written description. Applicants respectfully traverse this rejection.

Claim 1 is directed to a culture comprising a culture medium and a bacterium responsible for Whipple's disease. This bacterium is isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days. The recited bacterium is *Tropheryma whippelii* and has a doubling time

of 18 days. Applicants submit that the written description provides ample support for such a culture, which had never been obtained prior to the present invention.

The Patent Office relies on the holdings in University of California v. Eli Lilly and Co., 119 F.3d 1559 (Fed. Cir. 1997) and Enzo Biochem, Inc. v. Gen-Probe Inc., 323 F.3d 956 (Fed. Cir. 2002) in support of its rejection. However, both Eli Lilly and Enzo are directed to discoveries of genetic materials and uses thereof. Eli Lilly explained that a description of a broad genus of a genetic material, such as cDNA, may be achieved by means such as a recitation of structural features common to all members of the genus. 119 F.3d at 1569. In Enzo, the court held that reference in a patent specification to a deposit of genetic material may be sufficient to describe that genetic material. 323 F.3d at 970.

In contrast, claim 1 is not directed to a new genetic material, but is instead directed to a culture comprising a culture medium and a known bacterium responsible for Whipple's disease. Claim 1 is not directed to a newly discovered genetic material, the subject of both Eli Lilly and Enzo, but is directed to the selection and combination of a specific type of culture medium with a known species of bacterium, which allows reproducible and detectable multiplication of that bacterium over time. Thus, Applicants submit that the holdings of Eli Lilly and Enzo are not relevant to claim 1 because claim 1 is directed to a culture involving a known bacterium, and not a newly discovered genetic material as was the case in Eli Lilly and Enzo.

Contrary to the Patent Office's assertions, it is not necessary to provide more detail regarding the recited bacterium or to limit claim 1 to a specific deposited strain, because *Tropheryma whippelii* was known in the art as of the effective filing date of the present application. The specification only needs to describe in detail that which is new or not conventional. See MPEP §2163(II)(A)(3)(a). As acknowledged by Examiner Baskar during the December 8, 2006 interview, it has been known that a bacterium is involved with

Whipple's disease since 1907. See also, e.g., Maiwald, et al., "Cultivation of *Tropheryma whippelii* from Cerebrospinal Fluid," Journal of Infectious Disease, vol. 188, pp. 801-808 (September 15, 2003) (copy attached). As described in the specification, the species was observed by electron microscopy as early as 1961, and its phylogenic taxonomy was specified by 1992. Indeed, the bacterial species associated with Whipple's disease has been sequenced as early as 1991. See page 1, line 16 to page 2, line 8 of the specification. Thus, those of ordinary skill in the art know what is meant by *Tropheryma whippelii* and require no further written description of it. See, e.g., 323 F.3d at 965 (explaining that a biological deposit is not required to meet a written description requirement if the biological material is known).

Nor would persons of ordinary skill in the art have needed to read about any particular strains of *Tropheryma whippelii* or have required the deposit of any particular strains of *Tropheryma whippelii* in order to understand that the claimed invention had been fully described by the present specification. As discussed above, claim 1 focuses on the combination of a particular type of culture medium with the known *Tropheryma whippelii* bacterium. There is no suggestion, much less citation of evidence or reasonable scientific basis, in the Office Action that differences among strains of *Tropheryma whippelii* would have any effect on the practice of the invention of claim 1 or on the understanding of those of ordinary skill in the art that the invention of claim 1 had been fully described in the specification. The Office Action's statement that different strains would have different sequences and different proteins provides no suggestion that such differences would have been expected to have any effect on the practice of the invention of claim 1.

Further, antigen claim 10 and method claims 11 and 25 also are supported by an adequate written description in compliance with the written description requirement.

Claim 10 recites an antigen isolated from the *Tropheryma whippelii* bacterium in the culture according to claim 1. The specification clearly describes that the bacterium isolated

and established in culture as recited in claim 1 may be utilized as an antigen source. See page 3, lines 6-7 of the specification. It was well known in the art how to isolate an antigen once such a culture was obtained. Furthermore, an example of obtaining such an antigen is fully described in Example 6 of the specification. See pages 19-23 of the specification. Thus no further written description is required to support this claim either.

Claim 11 recites a method for the in vitro diagnosis of diseases associated with infection caused by *Tropheryma whippelii*, comprising contacting serum or any other biological fluid of a patient with a culture according to claim 1 or a *Tropheryma whippelii* bacterium obtained from said culture, and detecting an immunological reaction. Claim 25 recites a method for the in vitro diagnosis of diseases associated with infection caused by *Tropheryma whippelii*, comprising contacting serum or any other biological fluid of a patient with the antigen of claim 10, and detecting an immunological reaction. For the same reasons as discussed above with respect to claims 1 and 10, these claims also are supported by an adequate written description in compliance with the written description requirement.

In addition, Example 6 of the specification clearly describes how methods as recited in claims 11 and 25 were utilized for in vitro diagnosis of 15 patients. Further, the specification, beginning on page 4, clearly describes methods for the in vitro serological diagnosis of infections caused by *Tropheryma whippelii*, wherein the bacterium, an antigen of the bacterium or a specific antibody is brought into contact with a sample taken from the patient and consisting of a human serum, biological fluid or swab. Applicants thus submit that claims 11 and 25 also are supported by an adequate written description in compliance with the written description requirement.

Thus claims 1, 10, 11 and 25 are supported by an adequate written description in compliance with the written description requirement of §112, first paragraph. Dependent claims 15 and 29-32 are supported by an adequate written description for at least the same

reasons. The Office Action has not raised any additional bases for a written description rejection based on their additional limitations, and it is believed that there is no such additional basis.

In view of the foregoing, Applicants submit that claims 1, 10, 11, 15, 25 and 29-32 are supported by an adequate written description in compliance with the written description requirement of the first paragraph of 35 U.S.C. §112. Reconsideration and withdrawal of the written description rejection are thus respectfully requested.

B. Enablement

Claims 1, 10, 11, 15, 25 and 29-32 were rejected under 35 U.S.C. §112, first paragraph, as allegedly not being enabled by the specification. This rejection is respectfully traversed.

The Patent Office briefly stated that the present claims are rejected as lacking enablement because the specification allegedly fails to disclose the broadly claimed invention. See page 7 of the Office Action. The Patent Office provides no additional support or explanation of the enablement rejection.

First, the stated rationale is not a basis for an enablement rejection. The issue is whether one of ordinary skill in the art would have been able to practice the invention as claimed. There has been no allegation, much less presentation of evidence or scientific rationale, in the Office Action that such a person would not have been able to practice the invention as claimed. For this reason alone, the rejection should be withdrawn.

In addition, the present specification clearly discloses a culture comprising a culture medium and a bacterium responsible for Whipple's disease as required in claim 1. One of ordinary skill in the art would have been able to utilize the teachings of the present specification, including the examples, to create such a culture comprising a culture medium

and a bacterium responsible for Whipple's disease, such that the bacterium can be reproducibly and detectably multiplied over time in the culture medium.

For example, the specification explains that a cell culture in which *Tropheryma whippelii* is to be isolated and multiplied, must have both a long lifetime and a slow multiplication time, because the doubling time of the bacterium is very long, averaging 18 days. See page 2, lines 14-18 of the specification. Further, if the cells of such a culture multiply too rapidly relative to the growth of the bacterium, the bacterium cannot be cultivated because a dilution effect takes place and it becomes impossible to segregate the infected cells from the non-infected cells. See page 2, lines 25-27 of the specification. <u>Thus the specification provides sufficient detail such that one of ordinary skill in the art would have been enabled to make and use the culture recited in amended claim 1 given the information that is provided in the present specification, including the doubling time of the bacterium</u>. In fact, the record shows that those of ordinary skill in the art have actually been enabled by the Raoult disclosure to practice the invention. See, e.g., Bentely, et al., "Sequencing and Analysis of the Genome of the Whipple's Disease Bacterium *Tropheryma whipplei*," The Lancet, Vol. 361, pp. 637644 (February 22, 2003) (copy attached).

With respect to the antigen-related claims, one of ordinary skill in the art would have understood that every bacterium that is isolated and established in culture is also an antigen source. The present disclosure also describes use of the bacterium isolated and established in the culture of claim 1 as an antigen source, and identifies as exemplary antigens proteins selected from those with molecular weights of about 10, 20, 35, 50, 60, 80, 100, 120, 150, 170 and 200 kD determined by polyacrylamide gel electrophoresis. See, for example, page 3, lines 10-14 of the specification.

The specification further describes exemplary methods for the in vitro serological diagnosis of infection caused by *Tropheryma whippelii*, wherein the bacterium, an antigen of

the bacterium or a specific antibody is brought into contact with a sample taken from the patient and consisting of human serum, biological fluid or a swab. Such methods are described throughout the specification, for example at pages 4-8 of the specification.

Moreover, the Examples set forth in the specification are sufficiently detailed to ensure that one of ordinary skill in the art would have been enabled to practice the claimed invention.

For at least the foregoing reasons, Applicants submit that claims 1, 10, 11, 15, 25 and 29-32 are enabled by the specification. Reconsideration and withdrawal of the rejection are thus respectfully requested.

II. Rejections Under 35 U.S.C. §102(b)

A. Schoedon

Claim 1 was rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Schoedon et al., "Deactivation of Macrophages with Interleukin-4 is the Key to the Isolation of *Tropheryma whippelii*," The Journal of Infectious Diseases, 176:672-77 (1997) (hereinafter "Schoedon"). This rejection is respectfully traversed.

The Patent Office alleges that Schoedon teaches or suggests all of the features recited in claim 1. Applicants respectfully disagree.

Schoedon does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days, as required in claim 1.

Schoedon teaches a culture comprising a cell medium of monoblasts from the cell line SigM5, which is derived from bone marrow. See pages 673 and 674 of Schoedon. As is known to one of ordinary skill in the art, immortalized cell lines derived from monoblasts and monocytes have a very short doubling time. For example, mononuclear bone marrow cells

divide approximately six times during a culturing period of 14 days. See page 36 of Helin, et al., "Measles Virus Replication in Cells of Myelomonocytic Lineage is Dependent on Cellular Differentiation Stage," Virology, vol. 253, pp. 35-42 (1999) (copy attached). Further, it is known that cells from human promyelocytic leukemia cell line HL60 have a doubling time of 34.0 hours. See the Abstract of Foa, et al., "Growth Pattern of the Human Promyelocytic Leukemia Cell Line HL60," Cell Tissue Kinet. Vol. 15, no. 4, pp. 399-404 (July 1982) (copy attached).

Such a short doubling time of the cells in the culture medium taught by Schoedon would prevent the disclosed culture from being a culture as recited in claim 1. Specifically, due to the too short doubling time of the cells in the culture medium of Schoedon, these cells would double many times prior to the *Tropheryma whippelii* bacterium doubling even one time. Thus, the culture taught by Schoedon would not comprise a culture medium and bacterium that can reproducibly and <u>detectably</u> multiply in the culture medium for at least 72 days, as required in claim 1. To the contrary, the product of Schoedon would be diluted well below detectable limits in far less time.

One of ordinary skill in the art would have understood that the term "detectably" in the context of claim 1 reflects that the bacteria do not become increasingly diluted over time in the culture medium. In contrast, as demonstrated below, when the doubling time of cells in the culture medium is too short as in the applied references, the bacteria grown in such cells become dilute and undetectable over time.

This deficiency of Schoedon is graphically demonstrated in the Table below. This Table demonstrates the dilution effect when *Tropheryma whippelii* is cultured in a medium having a doubling time of two days (48 hours). A doubling time of two days exemplifies the outcome of culturing *Tropheryma whippelii* in a culture medium having a doubling time

significantly shorter than the doubling time (18 days) of the bacterium, yet still longer than the doubling time of the cells used in the Schoedon medium.

Day	Number of *Tropheryma whippelii* bacteria per number of cells in culture, where cells have a doubling time of 2 days
0	2 bacteria per 2 cells
2	2 bacteria per 4 cells
4	2 bacteria per 8 cells
6	2 bacteria per 16 cells
8	2 bacteria per 32 cells
10	2 bacteria per 64 cells
12	2 bacteria per 128 cells
14	2 bacteria per 256 cells
16	2 bacteria per 512 cells
18	2 bacteria per 512 cells
20	2 bacteria per 1024 cells

Extrapolating out to 72 days, it is readily apparent that a culture even in short-doubling-time cells with longer doubling times than those of Schoedon does not meet the requirements of claim 1.

Further, Schoedon teaches that the culture medium includes monocyte cells, which have a lifetime of 30 days. The present specification explains that this lifetime is insufficient in view of the doubling time of the bacterium. See page 2, lines 19-24 of the specification. Such a short lifetime of the Schoedon cell medium in combination with the short doubling time of the cells in the Schoedon medium, in comparison to the doubling time of the bacterium recited in claim 1 (18 days), indicates that the amount of bacteria in the cell medium of Schoedon is not sufficient to create a culture having a culture medium and a

bacterium that can reproducibly and detectably multiply over time in the culture medium for at least 72 days.[1]

During the December 8, 2006 interview, the Examiner suggested that Schoedon teaches that the cultures of *Tropheryma whippelii* in the culture medium are passaged every 8 to 10 days (see page 673, second column of Schoedon), and that allegedly there would be more *Tropheryma whippelii* bacterium present in the culture with each passage. Applicants strenuously disagree with this allegation. Specifically, as explained during the interview, with each passage of the culture medium taught by Schoedon, the cells of the culture medium would multiply while the number of bacteria present would remain constant. As such, over time, the bacterium in the culture would rapidly become <u>undetectable</u> because it would have been diluted by the too frequent passaging, especially coupled with the too-short doubling time of the cells in which the bacteria are grown.

Moreover, as explained in the Declaration Under 37 CFR §1.132 submitted September 9, 2005, Schoedon does not report the presence of *Tropheryma whippelii* after more than 4 passages, which corresponds to ten days. Thus, Schoedon does not teach or suggest a bacterium being isolated and established in culture such that the bacterium can reproducibly and <u>detectably multiply</u> over time in the culture medium <u>for at least 72 days</u>, as required in claim 1. To the contrary, Schoedon at best teaches that the bacteria rapidly become undetectable in the Schoedon medium, as discussed above.

Furthermore, Schoedon is not an enabling reference, and is thus improperly applied as an anticipatory reference. A patent claim cannot be anticipated by a prior art reference if the

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[1] Schoedon also states that macrophages were infected with *Tropheryma whippelii*. However, macrophages do not multiply, and have a lifetime shorter than two months. Thus they are also incapable of forming the culture of claim 1. See also Reference 9 in the attached Maiwald article where Zaaijer reference is characterized as disclosing that "*Tropheryma whippelii* is easily ingested by interleukin-4-deactivated macrophages, but does not multiply."

allegedly anticipatory disclosures cited as prior are not enabled. See, for example, Elan Pharm., Inc. v. Mayo Found. for Med. Educ. & Research, 346 F.3d 1051, 1054 (Fed. Cir. 2003). As evidenced by the Declaration Under 37 CFR §1.132 submitted September 9, 2005 and Maiwald, et al., it has not been possible to reproduce or confirm the teachings of Schoedon in subsequent studies. In fact, Schoedon authors themselves have referred to the absence of reliable cultures of *Tropheryma whippelii*, and have referred to it as "uncultivated" in the Hinrikson article of record and discussed in the Drancourt Declaration of record. In fact, it has been posited that Schoedon may not even have been observing *Tropheryma whippelii*. See, e.g., Relman, "Editorial: The Whipple Bacillus Lives (Ex Vivo)," The Journal of Infectious Diseases, Vol. 177, pp. 752-754 (1997) (copy attached).

Thus, Schoedon does not teach or suggest a culture as recited in claim 1.

For at least the foregoing reasons, Schoedon does not teach or suggest all of the features recited in amended claim 1. Reconsideration and withdrawal of the §102(b) rejection over Schoedon are thus respectfully requested.

B. Muller

Claims 1, 30 and 31 were rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Muller et al., "Cultivation of T. Whippelii From Peripheral Blood Mononuclear Cells," Gastroenterology, Vol. 116, No. 4, Part 2, Abstract 910 (1999) (hereinafter "Muller"). This rejection is respectfully traversed.

First, Muller was published less than one year before the effective U.S. filing date of the present application (and indeed after the earliest priority date for this application), and thus is not prior art to the present application under 35 U.S.C. §102(b). Thus this rejection must be withdrawn.

Second, the Patent Office alleges that Muller teaches all of the features recited in claims 1, 30 and 31. Applicants respectfully disagree with this allegation.

Muller teaches a culture comprising a culture medium including cells of the monocyte cell line U937. Like Schoedon, Muller also teaches the use of a cell having a shorter doubling time than the doubling time of *Tropheryma whippelii*. As explained above and as demonstrated by the Table set forth above, such a discrepancy between the doubling time of the cells in the Muller culture medium and the doubling time of the *Tropheryma whippelii* causes the *Tropheryma whippelii* to rapidly become dilute and undetectable in the culture medium over time. Thus, like Schoedon, Muller fails to teach or suggest a culture comprising a culture medium and a bacterium, such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days, as required in claim 1.

Muller further teaches that *Tropheryma whippelii* from peripheral blood mononuclear cells were cultivated without antibiotics with IL-4 for 10 days. In other words, Muller teaches that the bacterium *Tropheryma whippelii* was isolated and detectable for only 10 days.

Applicants thus submit that Muller does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days. In contrast, Muller teaches that the bacterium is cultured in its medium for a mere 10 days. Applicants thus submit that one of ordinary skill in the art would not have looked to the teachings of Muller to obtain a culture comprising a culture medium and a bacterium responsible for Whipple's disease, such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days.

Moreover, as explained above, Muller teaches that the culture medium includes immortalized monocyte cells, i.e., monocyte cell line U937. Thus, Muller also does not teach or suggest that the culture is not a cell culture in monocyte cells, as recited in claim 30, or that

the culture is a cell culture in immortalized cells other than monocyte cells, as recited in claim 31.

For at least the foregoing reasons, Muller does not teach or suggest all of the features recited in any of claims 1, 30 and 31. Reconsideration and withdrawal of the §102(b) rejection over Muller are thus respectfully requested.

C. Drancourt

Claims 1, 30 and 31 were rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Drancourt, La Presse Medicale, Vol. 28: No. 8, pp. 435-39 (Feb. 27, 1999) (hereinafter "Drancourt"). This rejection is respectfully traversed.

The Patent Office alleges that Drancourt teaches or suggest all of the features recited in claims 1, 30 and 31. Applicants respectfully disagree.

As previously discussed and set forth in the Declaration of Professor Drancourt, the author of the Drancourt article, filed on September 9, 2005, the Drancourt article merely provides a summary of various articles concerning *Tropheryma whippelii*. It does not set forth the results of any additional experimentation. In particular, the only article relating to cultivation of *Tropheryma whippelii* mentioned in Drancourt, Reference No. 13, is the Schoedon reference discussed above. Thus Drancourt adds nothing to the teachings of Schoedon, discussed above.

As confirmed by Professor Drancourt himself in the Declaration of record, the Drancourt reference does not reflect any experimentation that was conducted to confirm the accuracy or repeatability of the work described in Schoedon. Instead, this paper merely summarizes the statements of researchers in the field as reported in the scientific literature, including Schoedon. Even so, the Drancourt reference stated at page 9 of the translation that such work needs to be confirmed before being adopted. As detailed in the Drancourt Declaration, the Schoedon work has not been confirmed in spite of efforts in the art to

confirm it, and even the authors of the Schoedon reference were unable to come up with reliable cultures of *Tropheryma whippelii* in their later work.

Thus, Drancourt also fails to teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days, wherein the bacterium is *Tropheryma whippelii*, and the bacterium has a doubling time of 18 days, as recited in claim 1. It also fails to disclose the further limitations of claims 30 and 31, as the cited Schoedon culture attempts were in a monocyte medium.

For at least the foregoing reasons, Drancourt does not teach or suggest all of the features recited in any of claims 1, 30 and 31. Reconsideration and withdrawal of the §102(b) rejection over Drancourt are thus respectfully requested.

III. Rejection Under 35 U.S.C. §103(a)

Claims 1, 11, 15, 25 and 29-32 were rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Muller, Schoedon or Drancourt in view of Kent, Arch.Pathol.Lab.Med 104 (10), pp. 544-47 (1980) (hereinafter "Kent") and Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory 1988, Chapters 14/5/6 (hereinafter "Harlow and Lane"). This rejection is respectfully traversed.

As discussed above, Muller is not prior art to the present application. Thus reliance on it in the §103 rejection is improper.

Kent and Harlow and Lane do not remedy the deficiencies of Muller, Schoedon and Drancourt. In particular, Kent and Harlow and Lane also do not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days, wherein the

bacterium is *Tropheryma whippelii*, and the bacterium has a doubling time of 18 days, as recited in claim 1.

Moreover, none of the applied references teach or suggest (1) an antigen isolated from the *Tropheryma whippelii* bacterium in the culture according to claim 1, as recited in claim 10, (2) a method for the in vitro diagnosis of diseases associated with infection cased by *Tropheryma whippelii*, comprising contacting serum or any other biological fluid of a patient with a culture according to claim 1 or a *Tropheryma whippelii* bacterium obtained from such culture, and detecting an immunological reaction, as recited in claim 11, and (3) a method for the in vitro diagnosis of diseases associated with infections caused by *Tropheryma whippelii*, comprising contacting serum or any other biological fluid of a patient with the antigen of claim 10, and detecting an immunological reaction, as recited in claim 25.

Applicants submit that Muller, Schoedon, Drancourt, Kent and/or Harlow and Lane also do not teach or suggest features recited in claims 11, 15, 25 and 29-32, which directly or indirectly depend from claim 1.

The Office Action relies for support for the §103 rejection on a passing reference to "the readily available bacteria." However, because the art had not discovered how to make a culture in accordance with claim 1, there was no such "readily available" *Tropheryma whippelii* bacteria. While that bacteria could be identified in patient samples and materials infected with it, it could not be produced in amounts necessary for practice of the claimed methods or to produce the claimed isolated antigen. Thus, it would not have been obvious to practice such methods or produce such antigen.

Furthermore, the record is replete with evidence that the claimed invention not only would not have been obvious, but that it was not obvious to those of at least ordinary skill in the art. The record shows the existence of a very longfelt unsatisfied need for the claimed

invention, which others had tried and failed to achieve, that was first met by applicants by way of the claimed invention, resulting in much acclaim in the industry.

In Eli Lilly Co. v. Zenith Goldline Pharmaceuticals, Inc., Appeal No. 05-1396 (Fed Cir. December 26, 2006), the Federal Circuit held that establishing four of the five secondary considerations, including a long-felt need, failure of others, industry acclaim and unexpected results, strongly confirmed that the compounds that were the subject of dispute were not obvious in view of the prior art references. Similarly, the numerous articles presented to the Patent Office address many of the same "secondary considerations" exhibited by the presently claimed invention.

For example, Relman at page 752, first sentence, stated that "Cultivation of the bacillus associated with Whipple's disease, *Tropheryma whippelii*, has been an elusive goal for many generations of clinicians and microbiologists familiar with this disease." Maiwald at page 801, second column, confirmed that "the cultivation of this bacterium has been a goal of clinicians for several decades." Even the Drancourt reference applied in the Office Action refers to "90 years of isolation attempts" at page 9 of the translation. The Bentley and Maiwald references confirm that the present invention solved this longfelt need. See, e.g., Maiwald abstract and Bentley, page 637, col. 2. Maiwald even noted that Schoedon's teachings did not do so and were not reproducible. See page 802, first column of Maiwald. See also, the Drancourt Declaration of record. Thus the objective evidence of record is clear and convincing "secondary considerations" evidence of the non-obviousness of the claimed invention.

For at least the foregoing reasons, Applicants submit that Muller, Schoedon, Drancourt, Kent, and Harlow and Lane, in combination or alone, do not teach or suggest the subject matter of claims 1, 11, 15, 25 and 29-32. Reconsideration and withdrawal of the rejection are thus respectfully requested.

IV. New Claims 35-65

Applicants submit that none of the applied references teach or suggest the subject matter of new claims 35-65.

For example, none of the applied references teach or suggest that the bacterium is capable of reproducibly and detectably multiplying over time in said culture medium through successive subcultures, as recited in claim 38, or wherein the culture has been established in culture through successive subcultures, as recited in claim 39.

Moreover, none of the applied references teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days through successive subcultures, wherein the bacterium is of the same species as the *Tropheryma whippelii* bacterium strain deposited in the CNCM of the Institut Pasteur under Deposit No. I-2202 and has a doubling time of eighteen days wherein the bacterium comprises a rpoB gene comprising a partial sequence amplifiable by primers of a sequence identical to SEQ ID NO:4 or 5, as recited in claim 63.

Further, none of the references teach or suggest the processes of new process claims 46-62, 64 and 65, which were added in response to the examiner's suggestion. For example, none of the applied references teach or suggest (1) a process of culturing *Tropheryma whippelii* bacteria responsible for Whipple's disease, comprising isolating and establishing said bacteria in a culture medium such that said bacteria are capable of reproducibly and detectably multiplying over time in the culture medium for at least 72 days, as required in claim 46, (2) a process of culturing *Tropheryma whippelii* bacteria responsible for Whipple's disease, comprising isolating and establishing said bacteria in a culture medium such that said bacteria are capable of reproducibly and detectably multiplying over time in the culture

medium for at least 72 days through successive subcultures, wherein the bacteria comprise a rpoB gene comprising a partial sequence amplifiable by primers of a sequence identical to SEQ ID NO:4 or 5, and wherein the bacteria are of the same species as the *Tropheryma whippelii* bacterium strain deposited in the CNCM of the Institut Pasteur under Deposit No. I-2202 and has a doubling time of 18 days, as recited in claim 64, or (3) a process further comprising maintaining the bacteria in culture for at least 72 days, as recited in claims 62 and 65.

V. Conclusion

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance. Favorable reconsideration of the rejections, and prompt allowance of claims 1, 10, 11, 15, 25, 29-32 and 35-65, are earnestly solicited.

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Measles Virus Replication in Cells of Myelomonocytic Lineage Is Dependent on Cellular Differentiation Stage

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Measles virus (MV)-infected monocytes may have a central role in virus-induced immunosuppression. Our understanding of MV replication in monocytic cells is, however, incomplete. In this work we have investigated MV replication in cells of human myelomonocytic lineage with different maturation stages in order to study the effect of cellular maturation on virus infection. MV was able to infect human bone marrow myeloid granulocyte–macrophage colony-forming cells (CFC-GM) as well as monocytes and macrophages, but the replication cycle seemed to be regulated by the maturation stage of the cells. Virus infection in CFC-GM was productive, unlike in monocytes and macrophages, where an extensive viral RNA synthesis occurred and high amounts of proteins were synthesised without a remarkable release of infectious virus. Efficiency of viral macromolecular synthesis in macrophages was comparable to that of promonocytic cell line U-937 and human epithelial cell line A549, but in contrast to macrophages the cell lines highly supported productive infection. On the other hand, chemically induced maturation of the human promyelocytic and promonocytic cell lines HL-60, THP-1, and U-937 to more mature macrophage-like forms did not markedly alter the replication cycle of MV in these cell lines. Our results showed that MV replication in myelomonocytic cells varied depending on the maturation stage of the cells. The immature myelomonocytic cells supported productive virus infection, but the maturation process lead to cellular changes that caused a restriction of MV replication cycle partly at posttranscriptional and partly at posttranslational level. The metabolic milieu of monocytes and macrophages as such was sufficient to support extensive viral macromolecular synthesis.

Key Words: measles virus; myelomonocytic cells; bone marrow.

INTRODUCTION

Measles virus (MV), a single-stranded negative-sense RNA virus, is continuously globally an important pathogen causing 1 to 2 million annual deaths, mainly in developing countries. During acute illness, MV infects peripheral blood leukocytes (Berg and Rosenthal, 1961; Sullivan *et al.*, 1975) and monocytes are the major target cells (Salonen *et al.*, 1988; Esolen *et al.*, 1993). Infection causes strong immunosuppression with currently rather poorly known mechanisms. Infected monocytes may have a central role in the induction of immunosuppression. MV infection in monocytes changes many of their functions, e.g., by increasing production of interleukin-1β and reducing levels of tumour necrosis factor-α and interleukin-12 (Leopardi *et al.*, 1992; Ward *et al.*, 1991; Karp *et al.*, 1996). The expression of MHC class II molecules and the antigen-presenting function of HLA-DR, -DQ, and -DP molecules are enhanced in MV-infected monocytes *in vitro*, but no change is detected in infected promonocytic cell line THP-1, indicating that activation and/or maturation stage of the cells may play a pivotal role in virus-induced events (Leopardi *et al.*, 1993).

Besides disturbances caused by MV infection in immunological functions, infected cells of immune system can transport the virus to various target organs. MV replication in primary monocytes is highly restricted (Vainionpää *et al.*, 1991; Karp *et al.*, 1996), whereas immature cord blood monocytes from neonates support productive virus infection (Sullivan *et al.*, 1975). Moreover, the report by Bashle and co-workers (1985) suggests that MV replication may vary with the stage of differentiation or maturation of the cells. They have shown MV antigens in osteoclasts from patients with Paget's disease. Osteoclasts originate from bone marrow via mononuclear cells and have characteristics similar to macrophages. MV antigens were detected in both nuclei and cytoplasms of osteoclasts. This kind of intracellular distribution of MV antigens is known to be typical for persistently MV-infected cells (Norrby *et al.*, 1982; Chui *et al.*, 1986). Although an increasing number of studies indicate an important role of infected monocytes in MV immunopathogenesis, our knowledge about virus replication in myelomonocytic cells is limited. It is therefore of interest to analyse MV infection in more details in myelomonocytic cells with different maturation stages

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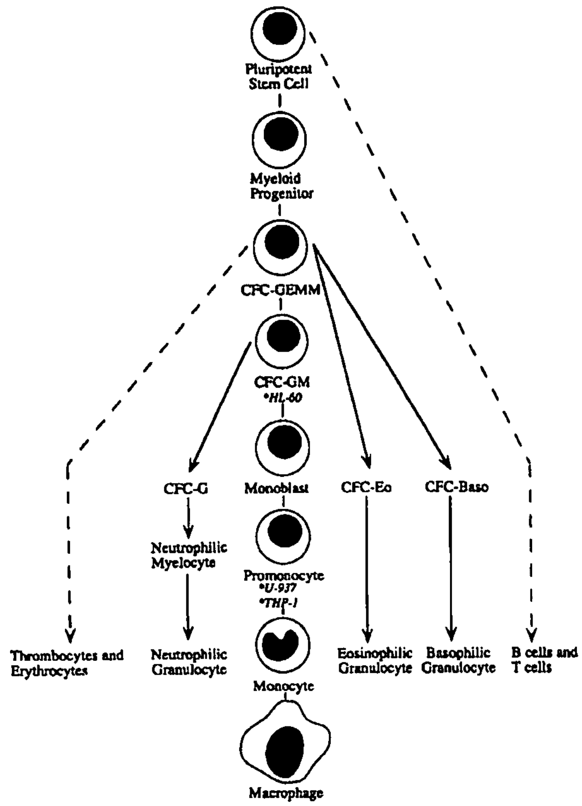

FIG. 1. Maturation and differentiation of myelomonocytic cells.

and study if host cell factors induced during maturation can modulate virus replication cycle.

To understand better MV infection in monocytic cells we have analysed MV replication in myelomonocytic cells with different maturation stages, from human bone marrow granulocyte/macrophage progenitors to monocytes and macrophages, as well as in promyelocytic and promonocytic cell lines HL-60, THP-1, and U-937. MV replication was productive in immature cells and restricted in mature monocytes/macrophages and the restriction occurred both at posttranscriptional and at posttranslational levels.

RESULTS

Maturation and differentiation of the cells of myelomonocytic lineage and the maturation stages of the promonocytic and promyelocytic cell lines THP-1, U-937, and HL-60 used in this study are shown in Fig. 1.

Myelomonocytic progenitors support productive MV replication

The mononuclear bone marrow cells divide approximately six times during the culturing period of 14 days, and granulocyte–macrophage colony-stimulating factor (GM-CSF) present in the culture medium supports exclusively the growth of only granulocyte–macrophage colony-forming cells (CFC-GM). Therefore, each colony is a product of one single progenitor cell and contains a mixture of original-type progenitor cells and more or less differentiated but still very premature cells of the myeloid lineage.

In contrast to mature monocytes, MV was able to replicate productively in progenitor cells of myelomonocytic lineage. The amount of infectious virus in culture medium increased from 8×10^1 PFU/ml (representing the rest of inoculum virus in medium after washings) to 3×10^3 PFU/ml (3 days p.i.) being at the same level still at day 5 p.i. Figure 2 shows a one-step growth curve of MV in CFC-GM. Immunofluorescent staining of the colonies showed sporadic MV antigen-positive cells, suggesting that infection was limited to a certain cell type (data not shown).

MV replication in monocytes/macrophages is restricted

We have earlier shown that MV replication in monocytes is incomplete. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) and Ca^{2+}-ionophore, which enhance protein kinase C activity and change a nonproductive MV replication to a productive one in peripheral blood mononuclear cells (PBMC), could not induce production of infectious virus in monocytes (Vainionpää *et al.*, 1991). In this study we examined whether differentiation of monocytes by IL-3 and/or GM-CSF would increase MV infection in monocytes/macrophages. For these experiments, monocytes were infected with MV, washed, and incubated for 3 days, after which maintenance medium was replaced by medium containing various concentrations of IL-3 (1, 10, or 25 IU/ml) and/or GM-CSF (10, 25, or 50 IU/ml). Incubation was continued for further 3 days and the specimens were collected for infectivity titration assay. Although more than 70% of monocytes in cultures contained intracellular MV proteins when examined by im-

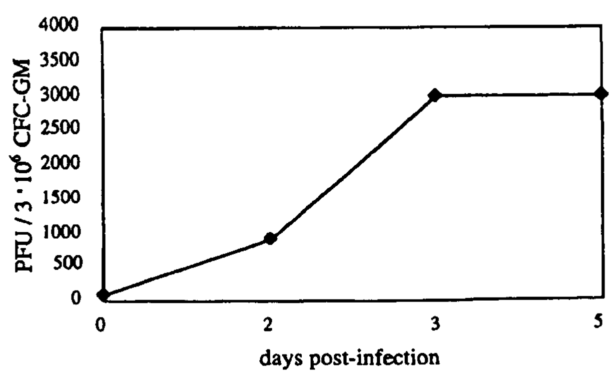

FIG. 2. One-step growth curve of measles virus in granulocyte–macrophage colony-forming cells (CFC-GM) quantified from the culture medium by plaque titration assay in Vero cells.

FIG. 3. MV infection of macrophages. Monocytes were infected with MV and then incubated in mitogen-stimulated medium for 3 days, after which the cells were fixed with 75% acetone and stained with the rabbit anti-measles virus serum and the HRP-conjugated goat anti-rabbit IgG. The figure shows one MV-infected, multinucleated macrophage and several smaller uninfected and infected mononucleated macrophages.

munofluorescent staining, neither the individual cytokines IL-3 or GM-CSF nor their mixture were able to increase MV replication (data not shown).

In order to further study the effect of maturation of monocytes to macrophages on their capacity to support MV replication, monocytes were infected, incubated for 3 days, and the maintenance medium was replaced by "mitogen-stimulated" medium or by medium from unstimulated PBMC culture. After 3 days the cells treated with mitogen-stimulated medium appeared as macrophage-like cells, and most of them were strongly positive for MV antigens when stained by the rabbit anti-measles virus antibodies. The cultures also contained MV antigen-positive multinucleated giant cells, one of which has been shown in Fig. 3. Maturation of infected monocytes to macrophages did not, however, change a restricted virus replication to a productive stage. Freezing and thawing did not have any effect on the release of infectious virus from macrophages either.

MV replication in monocyte-derived macrophage cultures was analysed in more detail by Northern and Western blotting techniques as well as by the infectivity titration assay. MV replication in the promonocytic cell line U-937 and in the epithelial cell line A549 was analysed for comparison. The macrophage cultures were maturated in macrophage SFM medium for 14 days as described under Materials and Methods. Figure 4 shows that virus RNA synthesis was more effective in macrophage cultures than the RNA synthesis in U-937 cells and comparable to the RNA synthesis in A549 cells, which are known to productively support MV replication. Viral proteins in macrophages were detected as well, indicating ongoing virus protein synthesis, although the protein amount was much lower than the proteins in the cell lines (Fig. 5). No release of infectious virus from macrophage cultures occurred, however. In contrast, virus replication in U-937 cells as well as in A549 cells was highly productive, the virus titres being 2×10^5 PFU/ml and 7×10^4 PFU/ml, respectively. These results showed that the metabolic milieu of monocytes/macrophages support MV macromolecule synthesis and the inhibition of effective virus production occurs at both posttranscriptional and posttranslational levels.

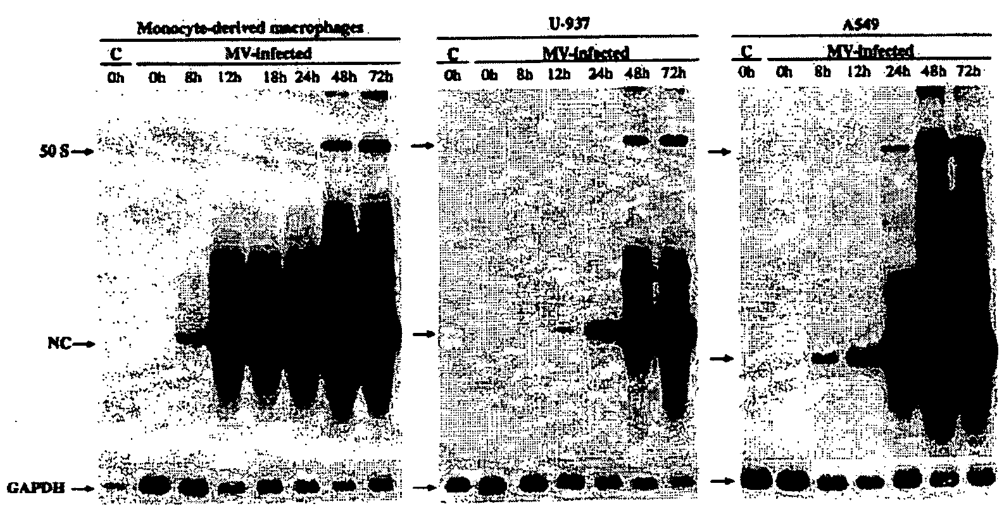

FIG. 4. Northern blotting analysis of total RNA in MV-infected monocyte-derived macrophages, U-937, and A549 cells. The specimens were collected at the times indicated. The filters were hybridised with the MV NC cDNA probe as well as with the GAPDH cDNA, which was used as an internal standard. The positions of the MV genomic size RNA 50S and NC are marked by the arrows. C, total RNA from uninfected cells.

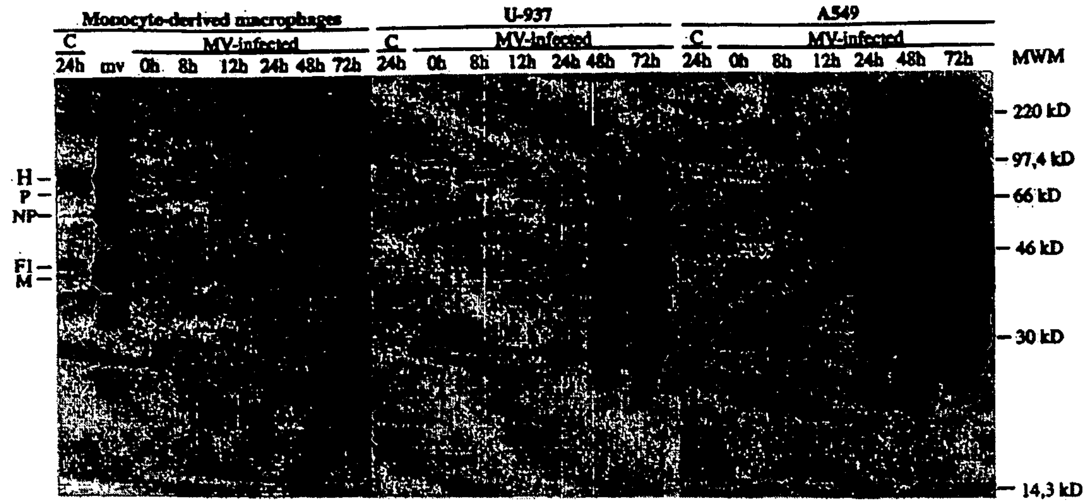

FIG. 5. Western immunoblot detection of MV proteins in monocyte-derived macrophages, U-937, and A549 cells. The specimens were collected at the times indicated, separated by SDS-PAGE, blotted onto nitrocellulose membrane, incubated with the rabbit anti-measles virus serum and the anti-rabbit HRP-conjugate, and detected by enhanced chemiluminescence. The positions of MV structural proteins are indicated at the left and the molecular weight markers at the right. mv, inoculum virus. C, uninfected cells.

MV infection in promyelocytic and promonocytic cell lines

For comparison, we studied MV replication also in the human promyelocytic and promonocytic cell lines HL-60, THP-1, and U-937. These cell lines, each of which represents a different stage of maturation, are widely used as *in vitro* models to study interactions between viruses and monocytes because they display a number of monocytic characteristics. They can also be induced to mature with phorbol esters such as TPA to more mature macrophage-like cells. HL-60 cells are closely related to early progenitor cells. They have bilinear differentiation potential and they can be chemically induced to differentiate to either granulocytic or monocytic cells. THP-1 and U-937 cell lines are from the monocyte/macrophage lineage and U-937 cells represent a later maturation stage. Chemically induced maturation has been reported to change the permissivity of these cell lines for replication of many other viruses (Roivainen and Hovi, 1989; Tenney and Morahan, 1991; Weinshenker *et al.*, 1988).

All three cell lines supported viral RNA synthesis to a similar extent (Fig. 6) when followed by spot hybridisation and measuring the radioactivity of the spots by liquid scintillation counting. However, the amount of released infectious virus did not correlate with the amount of viral RNA. The virus release was most effective in the promonocytic cell lines THP-1 (1×10^5 $TCID_{50}$) and U-937 (1×10^5 $TCID_{50}$). In contrast, the promyelocytic cell line HL-60 supported virus release only at a minimal level (5×10^1 $TCID_{50}$).

Because MV replication in PBMC is dependent on cellular activation stage (Lucas *et al.*, 1978; Hyypiä *et al.*, 1985), we wanted to compare the proliferation capacity of these cell lines by measuring their DNA synthesis by [^{3}H]thymidine incorporation. As shown in Fig. 7, THP-1 and U-937 cells were metabolically more active than HL-60 cells, and ongoing MV replication in these cells caused strong suppression of cellular DNA synthesis which was most evident in U-937 cells.

TPA is known to cause maturation of HL-60, U-937, and THP-1 cells (Tsuchiya *et al.*, 1982). In order to study the effect of maturation on MV replication, the cell lines were

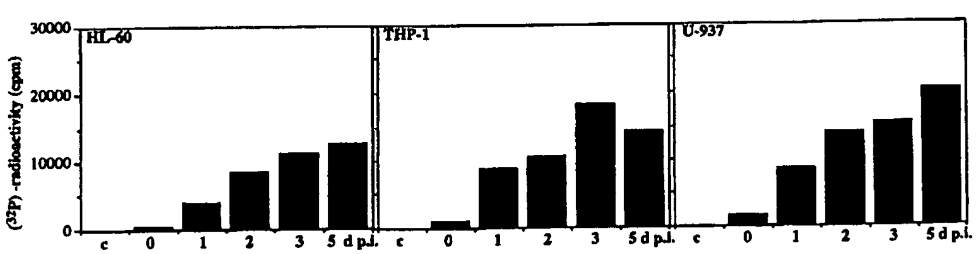

FIG. 6. MV-specific RNA synthesis in the promyelocytic and promonocytic cell lines HL-60, THP-1, and U-937 analysed by spot hybridisation and by measuring the radioactivity of the spots by liquid scintillation counting.

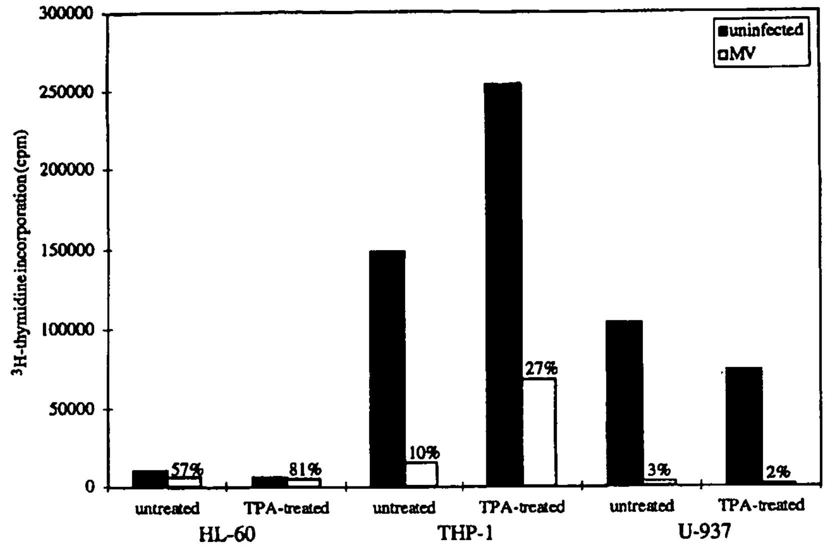

FIG. 7. DNA synthesis, as measured by [^{3}H]thymidine incorporation, in MV-infected and uninfected monocytic cell lines, which were either untreated or pretreated with TPA (1.0 nM) for 24 h before infection. The mean cpm (counts per minute) values of three parallel cultures are shown. The cpm values of MV-infected samples relative to corresponding uninfected samples are presented in percentages.

treated with 1 nM of TPA for 24 h before infection. The changes caused by maturation on cell proliferation were followed by measuring DNA synthesis by [^{3}H]thymidine incorporation (Fig. 7). TPA stimulated the proliferation of THP-1 cells, but caused suppression of DNA synthesis in HL-60 and U-937 cells. No clear-cut effect of cellular maturation on virus RNA synthesis as determined by spot hybridisation or on virus production was detected (data not shown). Our results showed that although TPA-induced maturation caused changes in cellular activity, no similar decrease/increase was observed in virus replication, indicating that MV replication did not depend entirely on the metabolic activity of the host cell.

DISCUSSION

Our results showed that human bone marrow progenitor cells were susceptible to productive MV replication. During the maturation of myelomonocytic cells some changes occurred, which lead to restriction of MV infection. Monocytes/macrophages supported extensive viral RNA and protein synthesis, but no clear-cut release of infectious virus was observed.

Infected monocytes may have a central role as mediators of immunosuppression, which is most probably a multifactorial event. On one hand, active virus replication is needed for suppression, because UV-inactivated virus is able to suppress cell proliferation in a much less extent than infectious virus does. On the other hand, interaction of MV glycoproteins with the surface of uninfected PBMC is sufficient to induce immunosuppression (Schlender *et al.*, 1996). Evidently cell cycle arrest of infected B- and T-lymphocytes is also partly responsible for suppression (McChesney *et al.*, 1987, 1988). MV is known to cause many cytokine dysfunctions of monocytes (Griffin and Ward, 1993), e.g., down-regulation of IL-12 (Karp *et al.*, 1996), which is known to be critical for the generation of cell-mediated immunity. This down-regulation was induced also by UV-inactivated virus. One obvious mechanism involved in immunosuppression is apoptosis observed in monocytes, dendritic cells, and T-lymphocytes in MV-infected cultures. MV-infected dendritic cells have been shown to induce apoptosis also in uninfected T-lymphocytes (Fugier-Vivier *et al.*, 1997).

As described above, an increasing amount of information suggests the central role of monocytic cells in MV immunopathogenesis, but much less is known about virus replication in myelomonocytic cells. Sullivan *et al.* (1975) have reported that the immature cord blood monocytes from neonates support a complete replication cycle of MV, and because a considerable proportion of monocytes in circulation can be immature, MV infection in these cells can contribute to the spreading of infection. An interesting question is whether MV can infect bone marrow progenitor cells. MV is known to cause life-long immunity, and it has been proposed that the persistence of the virus in bone marrow could be a reason for prolonged antibody production after primary infection. In this work we demonstrated that MV was able to infect bone marrow myeloid progenitor cells *in vitro*, and CFC-GM supported productive MV infection. Bone marrow cells are known to be affected in a number of different virus infections. For instance, human parvovirus B19 can cause aplastic crisis in patients with sickle cell anaemia (Pattison *et al.*, 1981), cytomegalovirus in-

fects both stromal and hematopoietic progenitor cells (Maciejewski *et al.*, 1992), and human herpes virus 6 (Knox and Carrigan, 1992) and dengue virus (Nakao *et al.*, 1989) infect bone marrow cells. To our knowledge this is the first time when MV has been shown to infect and replicate in human bone marrow progenitor cells.

There is variation in reports concerning MV replication in monocytes. Joseph *et al.* (1975) have described productive MV replication in monocytes, whereas we and others have shown that in mature monocytes/macrophages MV replication is highly restricted, and stimulation with various extracellular mitogens, which activate different biochemical pathways, does not activate the silent infection to a productive one (Vainionpää *et al.*, 1991; Karp *et al.*, 1996). This variation might be caused by different factors, e.g., maturation stage of monocytes. Also the susceptibility of monocytes from different individuals can vary.

In this report we have shown that the metabolic milieu of monocytes/macrophages as such could support MV macromolecular synthesis, because extensive viral RNA and protein synthesis occurred. The active virus protein synthesis, without release of infectious virus, in monocytes/macrophages may lead to accumulation of virus glycoproteins on cell surface, and this phenomenon could be an important factor in monocyte-mediated immunosuppressive events. Also the report by Bashle and co-workers (1985), describing persistent-type MV infection in osteoclasts, suggests restriction of MV replication in mature cells of the same myelomonocytic lineage. Cirino and co-workers (1993) have reported that respiratory syncytial virus (RSV), a member in the family of Paramyxoviridae, replicates productively in freshly isolated alveolar macrophages, but *in vitro* differentiation of monocytes into macrophages results in a significant, time-dependent decrease in production of infectious virus. The mechanisms by which cellular differentiation restricts MV and RSV replication are still unknown.

As a conclusion, our results suggest that the maturation stage of myelomonocytic cells may have an important role in the pathogenesis of measles. On one hand, immature monocytic cells can support productive virus replication, which can lead to the dissemination of the virus in the body. On the other hand, mature monocytes/macrophages support extensive virus RNA and protein synthesis, without release of infectious virus. These cells containing high amounts of virus proteins can be responsible for other dysfunctions, such as immunosuppression occurring in MV pathogenesis.

MATERIALS AND METHODS

Virus

A wild-type measles virus (Halonen-strain, Vainionpää *et al.*, 1978) with a high infectivity titre (>1 × 10^7 PFU/ml) was used throughout the study. The inoculum virus was propagated in Vero cells. For RNA and protein isolation the cells were infected at a multiplicity of infection (m.o.i.) of 5. In all the other works cells were infected at a m.o.i. of 1. After the adsorption time of 60 min those cultures later checked for virus production were thoroughly washed, and fresh maintenance medium was added.

Bone marrow progenitor cells

CFC-GM cultures were prepared as described earlier by Vuorinen *et al.* (1996). Briefly, mononuclear cells were obtained by the COBE 2991 Model I blood cell processor (COBE) from Ficoll–Paque (Pharmacia, Uppsala, Sweden)-separated heparinised bone marrow collected from autologous transplantation patients (disease-free at the time of the collection). The *in vitro* colonies of hematopoietic progenitors were cultured by the methyl cellulose technique originally developed by Pike and Robinson (1970) with the modification of Guilbert and Iscove (1976). Mononuclear bone marrow cells (2 × 10^5/ml) were mixed with culture medium containing 1% methyl cellulose, 20% foetal bovine serum (FBS, HyClone), 1% delipidated and deionised bovine serum albumin (Sigma, Cell Culture), 1 × 10^{-4} M β-mercaptoethanol, and 0.5 mg/ml fully iron-saturated human transferrin (Behringwerke) in Iscove's Modified Dulbecco's minimum essential medium (Gibco) and added to plastic Petri dishes. CFC-GM were cultured in the presence of GM-CSF (Leucomax, Sandoz, Schering-Plough), which supports the growth of only granulocytic and monocytic lineage cells. The plates were incubated for 14 days at 37°C in a fully humidified atmosphere with 5% CO_2.

The propagation of the bone marrow cultures was performed by Central Laboratory, Department of Haematology, Turku University Central Hospital to check the viability of autologous bone marrow transplantation material. The propagation took 2 weeks after which this anonymous waste material could be used for our purpose.

For virus infection the monocytoid colonies were picked under a light microscope, infected with MV (1 m.o.i.), washed, and suspended into the culture medium (without methyl cellulose) as described above.

Other cells and cell lines

Human peripheral blood puffy coat fractions of healthy blood donors were obtained from The Finnish Red Cross Blood Transfusion Service, Turku. To isolate mononuclear cells, the puffy coat cells were centrifuged at 1600 rpm for 45 min through a Ficoll–Paque cushion. The monocytes were enriched by adherence to polystyrene plastic plates, and nonadherent cells were removed by washing with Hanks' balanced salt solution. The cell preparations contained more than 90% monocytes as estimated by their light scattering properties in fluorescence-activated cell sorter analysis. The cells were

maintained at 37°C with 5% CO_2 in RPMI 1640 medium (Gibco) supplemented with 10% human AB serum; in MV-infected cultures 10% FBS was used instead of human serum.

Monocyte-derived macrophages were cultured from monocytes (isolated as described above) in macrophage SFM medium (Gibco) containing 10 ng/ml of GM-CSF for 14 days, during which period the medium was replaced every second day by fresh medium. Maturation of monocytes to macrophages was also done by incubating the MV-infected monocytes in mitogen-stimulated medium, which was a supernatant from concanavalin A (Con A)-treated uninfected PBMC. For preparation of such a supernatant, uninfected PBMC were treated with Con A (10 μg/ml) overnight, after which Con A was washed out with medium containing 0.3 M α-methyl-D-mannoside. Incubation was continued for 2 days, and the cells were pelleted and the supernatant was used as mitogen-stimulated medium. Maturation of the monocytes was detected based on morphology.

The human promyelocytic cell line HL-60 (ATCC CCL-240) and the promonocytic cell lines THP-1 (ATCC TIB-202) and U-937 (ATCC CRL-1593) were cultured in RPMI 1640 medium supplemented with 10% FBS. The maturation of the cell lines was induced by treatment with 1.0 nM of TPA (Sigma, St. Louis, MO) overnight. The human epithelial cell line A549 (ATCC CCC-185) was maintained in Ham's F-12 medium.

Assays for virus infectivity

The amount of infectious virus was determined by incubation of serial 10-fold dilutions of supernatants from infected cells on confluent monolayers of Vero cells. Each dilution was assayed in duplicate. After 5 to 7 days, the virus titre was determined after either a standard plaque counting (Vainionpää *et al.*, 1978) or a reading of the $TCID_{50}$ by light microscopy.

Immunostaining of cells

Immunoperoxidase staining of macrophages was done as described by Waris *et al.* (1990). Briefly, the cells were fixed on polystyrene plastic plates with cold 75% acetone and incubated first with the rabbit anti-measles virus serum and then with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories; diluted 1/400). 3-Amino-9-ethylcarbazole dissolved in dimethylformamide was used as chromogen.

Nucleic acid hybridisation

A cDNA clone composed of 1.6 kb of MV nucleoprotein mRNA in a pBR322 vector was used as a probe. Labelling of the probe, treatment of the cells, and hybridisation procedures were done as described by Hyypiä *et al.* (1985).

RNA isolation and Northern blotting

For RNA analysis, the specimens (10×10^6 cells) were harvested at indicated times. Total cellular RNA was isolated by guanidium isothiocyanate lysis followed by CsCl centrifugation 35,000 rpm overnight at 15°C (Chirgvin *et al.*, 1979). Equal amounts of RNA (20 μg) were electrophoresed in a 0.8% formaldehyde–agarose gel, transferred to a Zeta-Probe GT genomic tested blotting membrane (Bio-Rad Laboratories, CA) and hybridised with MV nucleocapsid-specific cDNA probe (a gift of Dr. T. Wong, University of Washington, Seattle, WA), labelled with [α-^{32}P]dCTP (Amersham) by a random primed DNA labelling kit. Determination of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by a cDNA probe (Fort *et al.*, 1985) was used as an internal control.

Immunoblotting

For virus protein analysis, the cells were lysed in RIPA buffer (50 mM Tris–HCl buffer, pH 8.0, containing 1% NP-40, 0.4% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin and aprotinin) and centrifuged 14,000 rpm for 30 min to remove insoluble material. Protein concentrations were estimated by Bio-Rad protein assay kit. The proteins (100 μg/lane) were separated by 10% SDS–PAGE, transferred electrically by Semi Dry Blot Pegasus (PHASE GmbH, Germany) onto Schleicher & Schuell nitrocellulose filter. After the blocking with 5% milk, the filters were incubated with the rabbit anti-measles virus antibodies (dilution 1:125 in phosphate-buffered saline containing 1% Triton X-100 and 5% milk powder) for 3 h at 37°C and then with the anti-rabbit HRP-conjugate. The bands were visualised by ECL chemiluminescence system (Amersham).

Cell proliferation assay

DNA synthesis of the untreated and TPA-maturated cell lines was measured by [^{3}H]thymidine incorporation. For maturation, the cells were treated with TPA at a concentration of 1 nM for 24 h before the infection and cultivated at a density of 1×10^5 cells/200 μl. [^{3}H]thymidine (Amersham; 0.5 μCi per well) was added 20 h before harvesting. The cells were harvested 4 days postinfection with a multichannel semiautomatic cell harvester (Skatron), and the radioactivity was measured in a 1217 Rackbeta liquid scintillation counter (Wallac, LKB).

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Should the Examiner believe that anything further would be desirable in order to place this application in even better condition for allowance, the Examiner is invited to contact the undersigned at the telephone number set forth below.

Respectfully submitted,

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Attachment:

Petition for Extension of Time

Maiwald, et al., "Cultivation of *Tropheryma whippelii* from Cerebrospinal Fluid," Journal of Infectious Disease, vol. 188, pp. 801-808 (September 15, 2003)

Helin, et al., "Measles Virus Replication in Cells of Myelomonocytic Lineage is Dependent on Cellular Differentiation Stage," Virology, vol. 253, pp. 35-42 (1999)

Abstract of Foa, et al., "Growth Pattern of the Human Promyelocytic Leukemia Cell Line HL60," Cell Tissue Kinet. Vol. 15, no. 4, pp. 399-404 (July 1982)

Bentley, et al., "Sequencing and Analysis of the Genome of the Whipple's Disease Bacterium *Tropheryma whipplei*," The Lancet, Vol. 361, pp. 637644 (February 22, 2003)

Relman, "Editorial: The Whipple Bacillus Lives (Ex Vivo)," The Journal of Infectious Diseases, Vol. 177, pp. 752-754 (1997) (copy attached)

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MAJOR ARTICLE

Cultivation of *Tropheryma whipplei* from Cerebrospinal Fluid

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(See the editorial by Scheld on pages 797–800.)

Whipple disease (WD) is a systemic disorder caused by the bacterium *Tropheryma whipplei*. Since the recognition of a bacterial etiology in 1961, many attempts have been made to cultivate this bacterium in vitro. It was eventually isolated, in 2000, from an infected heart valve, in coculture with human fibroblasts. Here we report the isolation of 2 new strains of *T. whipplei* from cerebrospinal fluid (CSF) of 2 patients with intestinal WD but no neurological signs or symptoms. One culture-positive specimen was obtained before treatment; the other was obtained 12 months after discontinuation of therapy, at a time of intestinal remission. In both cases, 15 passages of the cultures were completed over 17 months. Bacterial growth was measured by quantitative polymerase chain reaction, which suggested a generation time of 4 days. Staining with YO-PRO nucleic-acid dye showed characteristic rod-shaped bacteria arranged in chains. Fluorescent in situ hybridization with a *T. whipplei*–specific oligonucleotide probe, a broad-range bacterial probe, and a nonspecific nucleic-acid stain indicated that all visible bacteria were *T. whipplei*. Scanning electron microscopy and transmission electron microscopy showed both intracellular and extracellular bacteria. This first isolation of *T. whipplei* from CSF provides clear evidence of viable bacteria in the central nervous system in individuals with WD, even after prolonged antibiotic therapy.

In 1907, George H. Whipple described the postmortem examination of a patient who had died of a chronic disease presenting with arthritis, fever, weight loss, and cough [1]. He observed deposits of fat and fatty acids in the intestinal mucosa and mesenteric lymph nodes and named the disease "intestinal lipodystrophy." Whipple also observed small bacteria in silver-stained sections of a mesenteric lymph node, but he did not interpret this finding as causally related to the disease. Subsequent reports characterized Whipple disease (WD) as a rare, chronic, systemic disease, involving predominantly the intestinal tract but also a variety of other organs, especially the central nervous system (CNS) [2]. The etiology remained unclear for >40 years, until a bacterial cause was suggested by 2 observations: (1) a 1952 report of successful antibiotic treatment [3], and (2) the 1961 detection, by electron microscopy, of numerous, small, uniform bacteria in affected tissues [4, 5]. Both types of observations were subsequently confirmed and extended by many others.

Numerous attempts have been made to cultivate the WD bacterium in the laboratory, but they have either failed or yielded results that proved erroneous [2]. *Streptococcus* species, *Corynebacterium* species, and *Haemophilus* species are among the organisms so implicated [2]. Cultivation of this bacterium has therefore been a goal of clinicians and microbiologists for several decades. Characterization of the WD bacterium at the molecular level was accomplished during the early 1990s, by polymerase chain reaction (PCR) using broad-range primers

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to analyze bacterial 16S rDNA [6, 7]; analysis of the novel sequence established a phylogenetic relationship to the actinomycetes, and the name "*Tropheryma whippelii*" was proposed [7]. In 1997, on the basis of the notion that macrophages are the cell type most prominently involved in the pathology of WD, investigators inoculated interleukin-4–deactivated macrophages with heart-valve tissue affected by WD and reported propagation of bacteria [8]. However, this finding could not be confirmed in subsequent studies [9].

Long-term cocultivation of the WD bacterium with a human fibroblast cell line inoculated with heart-valve tissue was reported by Raoult et al. in 2000 [10]. The infection status of the fibroblasts was determined by microscopy, by periodic-acid–Schiff (PAS) staining, and by immunofluorescence, with the patient's serum. After inoculation of 1 cm^2 of cell monolayer, the cultures were expanded to 3750 cm^2 of infected cells over 7 passages within 9 months. After each passage, qualitative PCR detected DNA of the WD bacterium. The estimated bacterial doubling time was 18 days, which is longer than that of any other characterized bacterium. A second strain was subsequently isolated from a duodenal biopsy specimen [11], and the species designation was modified to "*whipplei*" [12]. Taken together, these reports provide good evidence for in vitro propagation of *T. whipplei*. Nonetheless, two important types of data are missing: (1) quantitative assessment of bacterial growth in vitro, by a molecular method, and (2) physical association of the *T. whipplei* 16S rRNA sequence with cultivated bacterial cells, by fluorescent in situ hybridization (FISH). The latter has been proposed as an important link between bacterial sequence and visible cells, especially when new taxa are described [13, 14]. Furthermore, the presence of viable *T. whipplei* bacteria has not been established in the CNS of individuals with WD. The availability of two cerebrospinal fluid (CSF) samples with large numbers of WD bacteria provided an opportunity to isolate new strains of *T. whipplei* and address all of these important issues.

PATIENTS, MATERIALS, AND METHODS

Patients and specimens. This work was approved by the Stanford University Administrative Panel on Human Subjects in Medical Research. CSF from 2 patients was used in these cultivation studies. Both patients presented with intestinal WD that was diagnosed by histopathology and by PCR analysis of *T. whipplei* 16S rDNA. Case 1 was a 74-year-old German man; the CSF specimen was obtained for the purpose of staging, before the initiation of therapy. The patient had no neurological symptoms or signs. Case 2 was a 52-year-old German woman; staging examinations by PCR analysis of *T. whipplei* in CSF [15] revealed CNS infection, but the patient had no neurological symptoms or signs. The patient was treated with an initial course of 2 weeks of penicillin plus streptomycin, followed by 1 year of oral cotrimoxazole. The CSF specimen used for culture was obtained for the purpose of monitoring response to therapy, 24 months after diagnosis and 12 months after discontinuation of antibiotics. At that time, results of PCR analysis of *T. whipplei* 16S rDNA of duodenal tissue were negative, and histology showed remission, in accordance with published criteria [16]. Diagnostic PCR analysis of *T. whipplei* 16S rDNA [15] showed strongly positive results for the CSF specimens from both patients, and the amplified sequence was completely homologous to the *T. whipplei* 16S rDNA (GenBank accession number X99636).

Cultivation methods. Cell cultivation on human fibroblasts was performed essentially as described elsewhere [10–12], with the following modifications: HEPES buffer (12.5 mM) was used in the medium, and fetal-calf-serum content was reduced from 10% to 1%, after confluent cell monolayers were obtained and before inoculation with bacteria. MRC-5 primary human embryonic lung fibroblasts (CCL-171; American Type Culture Collection) were cultivated in 25-cm^2 tissue-culture flasks (5-mL medium) and were inoculated with 500 μL of original CSF. Initial passages of the cultures were performed in 25-cm^2 flasks; 75-cm^2 flasks (25 mL) and 150-cm^2 flasks (35 mL) were later used for large-scale cultures. Each passage of the cultures involved inoculation of 20%–25% of the volume of supernatant onto new fibroblast monolayers after 4–6 weeks of incubation. Medium was changed infrequently: during the first passage, the medium was changed only after 3 weeks, and, during subsequent passages, the medium was either not changed or changed only after ~4 weeks of incubation. Beginning with the 13th passage, both MRC-5 cells and primary human foreskin fibroblasts (a gift from E. S. Mocarski, Stanford University) were used in parallel, for cultivation.

For quantitative measurement of bacterial growth, cell monolayers were cultivated in 6-well tissue-culture plates (9.5 cm^2/well) containing 2 mL of medium. On day 0, duplicate wells were inoculated with 0.5 mL of vigorously vortexed culture supernatant from a flask containing infected material. The contents of these wells were harvested on days 1 and 28 after inoculation: first, 1.25 mL of culture supernatant was removed, and then the cell monolayer was removed by a cell scraper and was harvested together with the residual 1.25 mL of supernatant. Both portions were frozen (−80°C) before analysis.

PCR. Tissue-culture supernatant or cell monolayers were centrifuged (18,000 *g* for 10 min), and DNA from the pellet was extracted as described elsewhere [15, 17]. To detect the presence of *T. whipplei* 16S rDNA, qualitative PCR using primers whip1 and whip2 [17] was performed; for bacterial identification, PCR using broad-range primers 8FPL plus 806R and 515FPL plus 1492RPL to analyze bacterial 16S rDNA [18]. Quantitative competitive PCR was performed according to

published protocols [19] and used the primers whip1 and whip2 [17] and a synthetic internal-standard molecule. This molecule (the "mimic") was constructed by PCR, according to instructions from the Clontech PCR mimic-construction kit. Composite primers were designed on the basis of the sequence of the *Bordetella bronchiseptica* filamentous hemagglutinin gene, *fhaB* [20], and *T. whipplei* 16S rDNA, so that the mimic consisted of a 217-bp sequence including whip1 and whip2 primer sequences at its ends. As a result, the mimic was easily distinguished, on the basis of size, from the 267-bp 16S rDNA amplification product (the "target") of *T. whipplei*. The product from *T. whipplei* and the mimic were each cloned into the TA vector (Invitrogen), plasmid DNA was extracted and quantified, and stock solutions containing 10^8 copies of each plasmid molecule/μL were prepared. Serial dilutions of the mimic molecule were used as internal standards in the PCRs, and serial dilutions of the *T. whipplei* product were used as quantitative references in control reactions. Samples from culture were initially tested against 10-fold dilutions of the "mimic," and then, for more accurate measurement, against 2-fold dilutions. The mimic concentration that, in agarose-gel electrophoresis, gave DNA-band intensity equal to that of the *T. whipplei* product was used to estimate the number of copies of *T. whipplei* rDNA in the sample.

Nucleic-acid staining. Nucleic acids in cultivated material were stained directly with YO-PRO-1 fluorescent dye (Molecular Probes). Culture supernatant was fixed in 3.7% formaldehyde, spotted onto glass slides, and air-dried. The slides were then overlaid with 2 μM YO-PRO-1 in water, incubated for 15 min, rinsed with water, immersed for 15 min in water, rinsed again, air-dried, and mounted with Vectashield mounting fluid (Vector Laboratories) and a coverslip (all steps were performed in the dark).

FISH. FISH was performed essentially as described elsewhere [21], with some modifications. In brief, culture supernatant was centrifuged (10,000 *g* for 10 min), and the pellets were resuspended in 1 × PBS, mixed with an equal volume of ethanol (final concentration, 50%), spotted onto Teflon-coated 10-well slides (Erie Scientific), and air-dried, at 45°C, on the wells. The samples on the slides were then fixed by incubations of 3 min each in 50%, 80%, and 96% ethanol. Hybridization was performed for 2 h at 46°C, with a solution containing 5 × SET, 1% SDS, 10% dextran, 0.2% bovine serum albumin, 0.1 mg polyadenosine/mL, and 5 μg of labeled probe/mL. The slides were then washed 3 times, for 10 min at 46°C, with 0.2 × SET at 46°C, rinsed with water, stained with 1 μM YO-PRO-1 in 1 × SET as described above, rinsed again, and mounted with Vectashield and coverslips. The following oligonucleotide probes were used: the *T. whipplei*–specific probe Tw16S-652 (5′-TTCCGCTCTCCCCTATCGCACTCT), the negative-control probe Tw16S-Cnt (5′-AAGGCGAGAGGGGATAGCGTGAGA) [21], the broad-range bacterial probe Eub16S-338 (5′-GCTGCCTCCCGTAGGAGT) [22], and the probe HGC69a (5′-TATAGTTACCACCGCCGT) for gram-positive bacteria with high G+C content [23]. Tw16S-652, Tw16S-Cnt, and HGC69a were labeled with the fluorophore Cy-3, and probe Eub16S-338 was labeled with Cy-5. Cultures of "*Corynebacterium aquaticum*" (ATCC 14665), *Cellulomonas cellulans* (ATCC 27402), and *Agromyces ramosus* (ATCC 25173)—all *Actinobacteria*—were used as bacterial controls. Slides were viewed and images were recorded by use of a BioRad MRC-1024 Laser Scanning Confocal Imaging System, as described elsewhere [21].

Electron microscopy. Cell monolayers were cultivated on round, 18-mm glass coverslips in 12-well (4-cm²) tissue-culture plates. Four weeks after inoculation, the medium was removed, and the cells were fixed, for 2 days, with 1.5% glutaraldehyde that was buffered to pH 7.3 by sodium cacodylate and that was made isotonic by the addition of sucrose. For scanning electron microscopy (SEM), the coverslips with cells and bacteria were dehydrated with alcohol and a critical-point bomb, were sputter-coated with 100-Å gold, and then were examined by use of an Hitachi S-2400 scanning electron microscope operating at an accelerating voltage of 15 kV. For transmission electron microscopy (TEM), the monolayers were postfixed, for 1 h, in 2% buffered osmic acid, dehydrated with alcohol, and embedded in epoxy resin. Sections were cut at 50-nm thickness, were stained serially with uranyl acetate and lead hydroxide, and then were examined by use of a Phillips 200 electron microscope operating at an accelerating voltage of 75 kV.

Strain deposition. The isolate from patient 2 (strain TW08/27) has been deposited in the American Type Culture Collection (ATCC culture number pending).

RESULTS

Six weeks after inoculation of MRC-5 primary human embryonic lung fibroblast monolayers with CSF from cases 1 and 2, qualitative PCR used to test for *T. whipplei* in culture supernatants from the 2 infected monolayers gave positive results. Cellular and bacterial material from 5 mL of supernatant was then concentrated, by centrifugation, in 1 mL and then was inoculated onto fresh monolayers in 25-cm² flasks. On days 1 and 15 after this passage, 100 μL of supernatant was collected and analyzed by quantitative PCR. A "low-resolution" quantitative-PCR analysis (using 10-fold dilutions of the mimic) indicated an increase in rDNA copy number, from 10^5/mL (CSF of case 1) and $<10^5$/mL (CSF of case 2) on day 1 to $\geq 10^6$/mL (in both cases) on day 15. Before inoculation, the original CSF specimens had shown copy numbers of 10^4/mL (case 1) and $<10^4$/mL (case 2), by the same PCR. In the subsequent, similar passage, supernatant from both cultures was stained with YO-PRO and showed small, rod-shaped bacteria in a characteristic

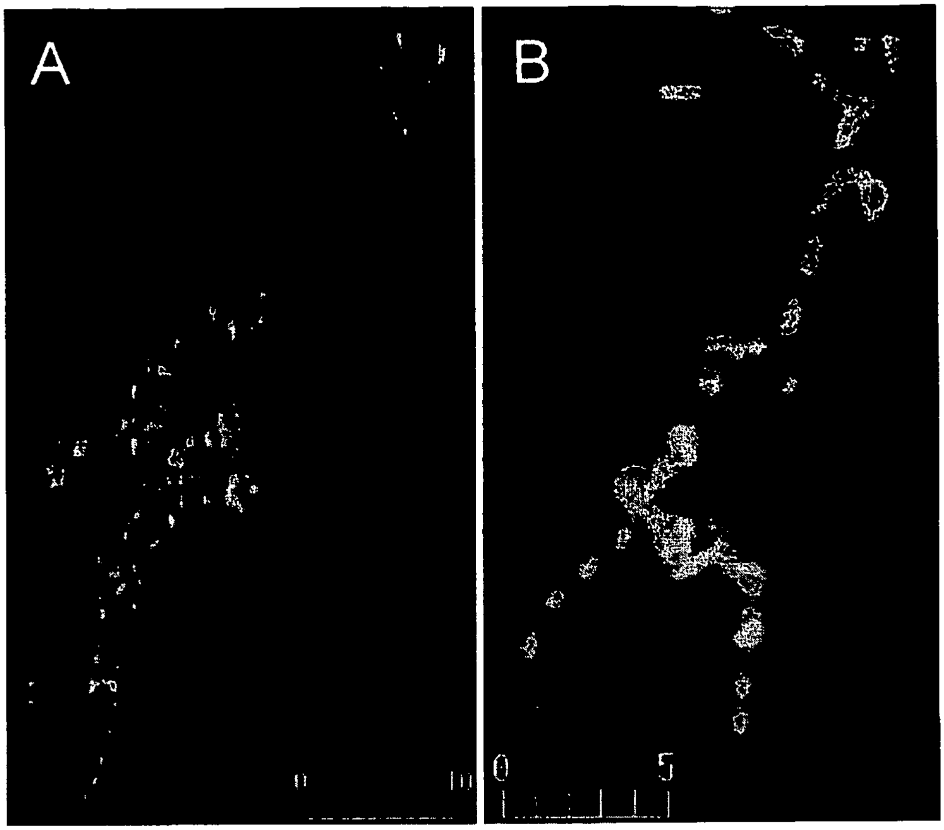

Figure 1. Photomicrograph of culture supernatant stained with YO-PRO nucleic-acids dye. *A*, Strain TW09/02 in third passage (original magnification, ×3000). *B*, Strain TW08/27 in ninth passage (original magnification, ×5000). Scale bars represent micrometers.

chainlike arrangement (figure 1*A*). Culture supernatant was also examined by PCR using broad-range primers to analyze bacterial 16S rDNA, in an assay that targets a 1443-bp region of the 16S rDNA. Direct sequencing of PCR products revealed unambiguous readings; the sequence from case 1 was a perfect match to that of *T. whipplei* [24]; the products from case 2 had only 2 nucleotide mismatches, in positions where they would not affect the 16S rRNA structure.

A total of 15 passages were performed with both cultures, over a period of 17 months. Beginning with the 13th passage, human foreskin fibroblasts were used in parallel with MRC-5 cells, because they appeared to form more-coherent monolayers and remained morphologically unaltered over longer incubation times. Cultures were regularly checked for the presence of bacteria, by staining the supernatant with YO-PRO; this was done at each passage, usually between the fourth week of incubation and the time of transfer to a new cell monolayer, and showed characteristic-looking bacteria (figure 1*B*). The strain from case 1 was designated "TW09/02," the strain from case 2 "TW08/27." The cultures were expanded to 40 flasks (150 cm^2 each) for strain TW09/02 and to 60 flasks for strain TW08/27. PCR analysis of broad-range bacterial 16S rDNA was repeated with both strains after their 15th passage, with the same results. Material from the 60 flasks with strain TW08/27 was harvested, and bacterial DNA was extracted and used for a genome-sequencing project [25] (see the http://www.sanger.ac.uk/Projects/T_whipplei/ Web site).

Quantitative-PCR studies (see Patients, Materials, and Methods) were performed again after the 11th passage, using 10-fold and, subsequently, 2-fold dilutions of the mimic (figure 2). Data from supernatants and data from combined fractions (supernatant plus cell monolayer) harvested on days 1 and 28 after inoculation were compared (table 1). *T. whipplei* 16S rDNA copy numbers were ~100-fold greater on day 28 than they were on day 1. In addition, rDNA copy numbers in the combined fractions were ~10-fold greater than those measured in the supernatants alone. These data suggest that, on average, the bacteria have completed 7 divisions during the intervening

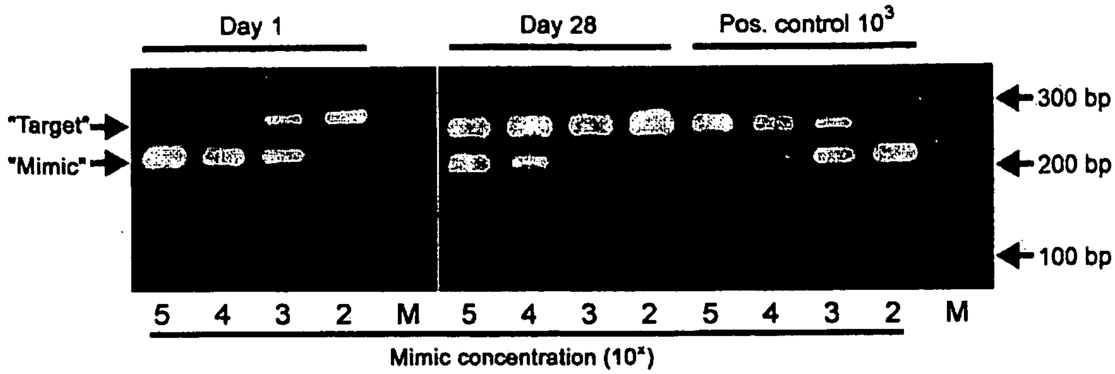

Figure 2. Agarose gel showing results of polymerase chain reaction (PCR) in cultures in 6-well plates (see Patients, Materials, and Methods), for combined fractions (supernatant plus cell monolayer) from strain TW08/27, on days 1 and 28 of incubation, tested against initial 10-fold dilutions of the "mimic" molecule. A positive control with the cloned *Tropheryma whipplei* "target" (10^3 copies) was also used. The copy numbers of mimics and targets used in a 50-μL PCR are given. Because of dilution factors, the calculated copy number per milliliter of culture material (table 1) is 1.5 log higher than the copy number used in a 50-μL PCR. M, molecular-weight marker.

27 days, which corresponds to a bacterial generation time of ~4 days. To confirm the specificity of the quantitative-PCR results, a target band from this assay was sequenced, for both strains; this sequence was identical to the 16S rRNA sequence of *T. whipplei*.

For both strains, FISH experiments with culture supernatant were performed after the 12th and 15th passages. All visible bacteria in supernatants hybridized with the *T. whipplei*–specific probe Tw16S-652, the broad-range bacterial probe Eub16S-338, and the actinobacterial probe HGC69a but not with the negative-control probe Tw16S-Cnt. All bacterial control strains hybridized with Eub16S-338 and HGC69a, none hybridized with Tw16S-Cnt, and only "*C. aquaticum*" hybridized, very faintly, with Tw16S-652, as described elsewhere [21]; this faint signal was easily distinguishable from the much-brighter signal in the 2 CSF cultures. Triple-label experiments, with YO-PRO, Tw16S-652, and Eub16S-338, revealed colocalized staining patterns with the 3 labels, for all bacteria in both cultures (figure 3), indicating a homogenous population of (*T. whipplei*) bacteria.

Electron microscopy of culture material from both strains was performed after the 14th passage. SEM showed intact extracellular bacteria (figure 4*A*), and TEM showed well-preserved bacteria both in extracellular locations and within the cytoplasm of healthy-appearing fibroblasts (figure 4*B*).

DISCUSSION

The results of the present study indicate that viable *T. whipplei* strains are found in the CSF of patients with WD and that they can be propagated in the presence of human fibroblasts in culture. These data confirm and expand on the findings reported by Raoult et al. [10–12]. They also provide the first quantitative measurement of the growth of *T. whipplei* in vitro. A previous report [8], describing the growth of *T. whipplei* in interleukin-4–deactivated macrophages, has been not confirmed, either by us (M.M. and D.A.R., unpublished results) or by other investigators [9].

Our data also document the first cultivation of *T. whipplei* from CSF samples. CSF is ideally suited for such studies, since it is a relatively simple fluid that is normally sterile. The examination of CSF has special relevance for diagnostic testing for WD, because (1) bacteria appear to invade the CNS early in the disease and (2) late manifestations affecting the CNS pose a significant threat to patients [2, 15]. This is illustrated by a number of published cases with symptomatic CNS disease, cases in which bacteria appeared to have been eradicated from the intestinal mucosa after therapy [2, 15, 17, 26–29]. One noteworthy case presented with severe insomnia as the only symptom 8 years after intestinal WD had been diagnosed and treated; at that time, results of intestinal/histological examination and PCR analysis of intestinal tissue were negative but PCR analysis showed that CSF was positive for *T. whipplei* [29].

Table 1. Results of quantitative polymerase chain reaction, for the 2 *Tropheryma whipplei* strains from cultures in 6-well plates (see Patients, Materials, and Methods).

	Strain TW09/02		Strain TW08/27	
Day after inoculation	Supernatant	Combined fraction	Supernatant	Combined fraction
1	5×10^3/mL	5×10^4/mL	$<5 \times 10^3$/mL	2×10^4/mL
28	8×10^5/mL	8×10^6/mL	5×10^5/mL	5×10^6/mL

NOTE. Both the culture supernatants (1.25 mL) and the combined fractions (1.25 mL), the latter of which consisted of supernatant plus cell monolayer, were tested during the 11th passage on each of the 2 days.

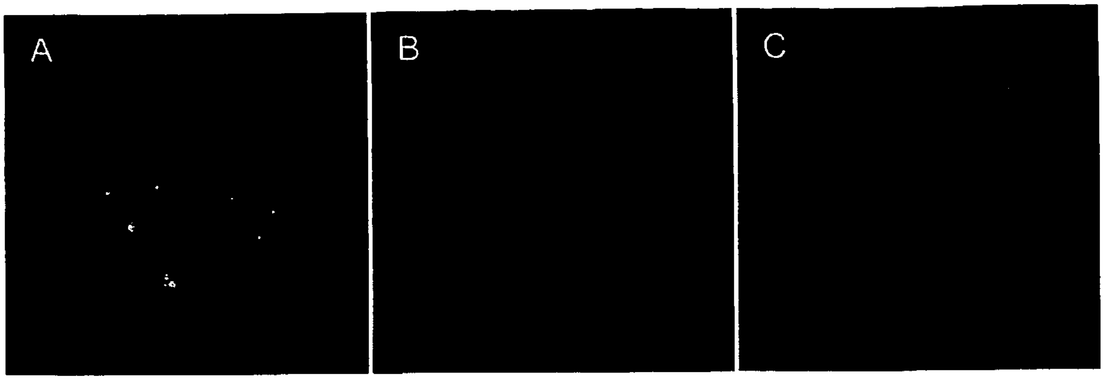

Figure 3. Photomicrographs after fluorescent in situ hybridization with strain TW08/27 from the 14th passage of the cultures, for dual hybridization with probes Tw16S-652 and Eub16S-338, followed by YO-PRO stain. *A*, YO-PRO stain (nonspecific DNA stain) viewed with the fluorescein isothiocyanate channel. *B*, Probe Tw16S-652 (*Tropheryma whipplei* specific) viewed with the Texas Red channel. *C*, Probe Eub16S-338 (bacterial broad range) viewed with the Cy-5 channel (original magnification, ×2000). Scale bar represents micrometers.

Often, manifestations of *T. whipplei* in CNS respond only partially to antibiotics and have a poor prognosis.

A previous study examined CSF samples from 24 patients with WD that were obtained at various times before and after therapy [15]; even in neurologically asymptomatic patients, PCR results were positive for the presence of *T. whipplei* in 7 of 10 cases before therapy and in 3 of 11 cases after therapy. These data indicate that the bacterium or its components are commonly present in the CNS of patients with intestinal WD and that, even in the presence of prolonged therapy with antibiotics, bacterial clearance may be delayed or uncertain. Furthermore, the data underscore the importance of using antibiotics that cross the blood-brain barrier. The isolation of 2 *T. whipplei* strains from CSF supports these concepts and emphasizes the importance of PCR-based, sensitive approaches for the detection and monitoring of CNS infection. The present study provides new evidence of viable *T. whipplei* in the CNS of patients with WD, even in the absence of neurological symptoms, and demonstrates that the bacterium can persist in a viable state, even after 1 year of therapy and intestinal-disease remission.

Quantitative measurement of bacterial growth is an important contribution to the evolving story of the propagation of *T. whipplei* ex vivo. The use of an internal standard (i.e., a mimic) avoids the potential problems of other types of PCR assays, in which PCR inhibitors might interfere with quantification [19]. Our calculated doubling time of 4 days differs from the previously reported time of 18 days, which was based on semiquantitative microscopic assessment of inclusions in fibroblast monolayers, inclusions that were shown to be positive for *T. whipplei* [10] when the PAS reagent was used, but it is still among the longest observed doubling times for any bacteria. This difference might be due either to the different measurement methods or culture conditions or to the differences between *T. whipplei* strains. Knowledge of the generation time is clinically relevant; with a doubling time of 4 days, a typical, 14-d intravenous therapy–induction period [30] spans only 3 replication cycles and thus might have to be reconsidered.

Bacterial morphology and the chainlike arrangement were distinctive when revealed by YO-PRO staining (figure 1). FISH now integrates, for the first time, bacterial morphology and the 16S rRNA sequence of *T. whipplei*. A previous study with sections from intestinal biopsy specimens did not resolve individual bacteria, probably because of high bacterial density and the thickness of the sections [21]. Triple-label experiments in the present study (figure 3) showed that nonspecific staining of DNA by YO-PRO, a broad-range bacterial probe, and a WD-specific probe all colocalized to the same bacterial shapes. These data and the absence of ambiguities in the PCR-based analysis of broad-range bacterial 16S rDNA performed during the third and 15th passages indicate that the cultures were not contaminated with other bacteria. Multiple FISH experiments clearly showed small, rod-shaped bacteria, but the slender shapes and the chainlike arrangement were not as well preserved as were those seen in staining by YO-PRO. The different morphologies seen by these 2 methods may arise from the different fixation procedures (i.e., formalin vs. alcohol) and/or the additional processing steps employed in the FISH protocol.

Uncertainty remains as to whether *T. whipplei* prefers intra- or extracellular growth environments. A detailed electron-microscopic study of intestinal WD [31] demonstrated that the majority of morphologically intact bacteria were located extracellularly in the lamina propria and that intracellular bacteria were in various stages of degradation. These findings are consistent with the results of more-recent work, which used FISH in intestinal biopsies [21] and which found *T. whipplei*–rRNA

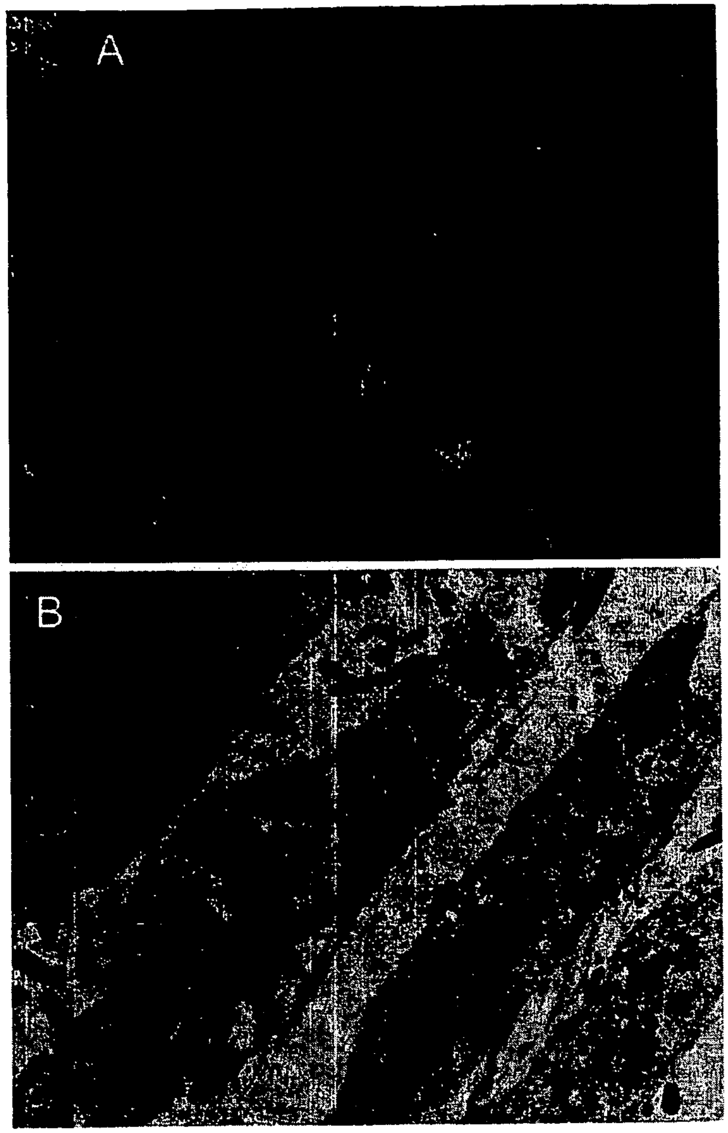

Figure 4. Electron micrographs of *Tropheryma whipplei* in fibroblast cell culture after the 14th passage. *A*, Results of scanning electron microscopy of strain TW08/27 (original magnification, ×20,000). *B*, Results of transmission electron microscopy of strain TW09/04 (original magnification, ×12,275).

hybridization signals, corresponding to metabolically active bacteria, in the lamina propria, directly subjacent to the epithelial basement membrane, but not inside cells. The location of the rRNA signal did not correspond to the inclusions characteristic of macrophages from patients with WD, inclusions that PAS shows to be positive for *T. whipplei*. On the other hand, Raoult et al. [10] reported intracellular growth in their fibroblast cell–culture system, which used PAS and immunofluorescence staining. In the present study, quantitative PCR with supernatant and with combined fractions indicated that *T. whipplei* grows in close association with fibroblasts but also grows in the cell-free supernatant. SEM clearly showed bacteria in extracellular locations (figure 4*A*); on the other hand, TEM showed intact bacteria in both intra- and extracellular locations (figure 4*B*). The host cells too appeared to be intact, and this obvious lack of cell damage is reminiscent of the paucity, in *T. whipplei* infection in humans, of both cell damage and inflammatory cellular infiltrate [16].

The *T. whipplei* isolate TW08/27 has been subjected to complete-genome sequencing [25] (see the http://www.sanger.ac.uk/Projects/T_whipplei/ Web site). Among fastidious and cultivation-resistant bacterial pathogens, the genome of *T. whipplei* is the third to be sequenced, after those of *Treponema pallidum* and *Mycobacterium leprae*. Although resistance to cultivation is uncommon among known pathogenic bacteria, the vast majority of bacteria in natural environments and in the commensal flora have not been cultivated in vitro [32, 33]. The extent to which currently uncharacterized or uncultivated bacteria might be involved in chronic idiopathic diseases is unclear [34]. In this context, *T. whipplei* is an attractive model organism with which to study such questions and, thus, to gather insight into related, important biological principles.

Acknowledgments

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Growth pattern of the human promyelocytic leukaemia cell line HL60. Foa P, Maiolo AT, Lombardi L, Toivonen H, Rytomaa T, Polli EE. Cell Tissue Kinet. 1982 Jul;15(4):399-404

In order to characterize the growth pattern of the human promyelocytic leukaemia cell line HL60, its kinetic parameters were studied. The doubling time was calculated from serial cell counts, the duration of the various cell cycle phases from the analysis of the labelled mitoses curve, and quiescent population from continuous labelling experiments. Proliferation in culture was exponential up to a saturation density of about 3.0 x 10(6) cells/ml, with a doubling time of 34.0 hr. The cell cycle duration was 24.3 +/- 4.1 hr (SD), and that of the cell cycle phases was: G1, 3.8 +/- 2.2 hr; S, 15.1 +/- 3 hr; and G2, 4 +/0 1.2 hr. The growth fraction was 0.85, and cell loss was restricted to the quiescent cells. The HL60 cell line, with fully characterized kinetics, provides a useful tool for the vitro study of substances which may affect human leukaemic myelopoietic proliferation.

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Articles

Sequencing and analysis of the genome of the Whipple's disease bacterium *Tropheryma whipplei*

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Summary

Background Whipple's disease is a rare multisystem chronic infection, involving the intestinal tract as well as various other organs. The causative agent, *Tropheryma whipplei*, is a Gram-positive bacterium about which little is known. Our aim was to investigate the biology of this organism by generating and analysing the complete DNA sequence of its genome.

Methods We isolated and propagated *T whipplei* strain TW08/27 from the cerebrospinal fluid of a patient diagnosed with Whipple's disease. We generated the complete sequence of the genome by the whole genome shotgun method, and analysed it with a combination of automatic and manual bioinformatic techniques.

Findings Sequencing revealed a condensed 925 938 bp genome with a lack of key biosynthetic pathways and a reduced capacity for energy metabolism. A family of large surface proteins was identified, some associated with large amounts of non-coding repetitive DNA, and an unexpected degree of sequence variation.

Interpretation The genome reduction and lack of metabolic capabilities point to a host-restricted lifestyle for the organism. The sequence variation indicates both known and novel mechanisms for the elaboration and variation of surface structures, and suggests that immune evasion and host interaction play an important part in the lifestyle of this persistent bacterial pathogen.

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Introduction

First described in 1907, Whipple's disease is a multisystem disorder, involving the intestinal tract and various other organs.[1] The disease is fatal if left untreated. The clinical presentation is heterogeneous. Frequently, patients complain of arthralgias, chronic diarrhoea, and weight loss, and less often from central nervous or cardiac manifestations, for years before diagnosis. Because of its varied manifestations, the disease has found its way into the differential diagnosis of many clinical disorders.

Since the 1960s, electron microscopy studies have consistently shown small, uniform, rod-shaped bacteria in affected tissues, measuring about 0·2×1·5–2·5 μm.[2] The bacterial cell wall has a trilaminar appearance, with an outer membrane that is proposed to be of host origin. Many attempts were undertaken to propagate this bacterium in the laboratory, but it proved resistant over many decades to cultivation. Broad-range bacterial 16S rDNA PCR followed by phylogenetic analysis[3,4] has placed the bacterium within the Gram-positive bacteria with high G+C content (class actinobacteria). The bacterium holds an intermediate position between actinobacteria with the common group A and the uncommon group B peptidoglycan, and is not closely related (16S rDNA divergence >7%) to any cultivated representative.[5] Isolation of the bacterium *Tropheryma whipplei* was achieved in 2000, in a long-term culture system with human fibroblasts, with a reported generation time of 18 days.[6]

Many aspects of Whipple's disease and *T whipplei* remain poorly understood, including clinical, histological, and epidemiological features of the illness, and metabolic capabilities, ecology, and interactions of the bacterium with the human host. Among the observations and unproven propositions are a close association of this bacterium with human beings, a possible bacterial environmental niche,[7] and a predilection for causing disease in outdoor workers.[1] Some have proposed that patients with Whipple's disease have subtle immune defects.[8] Additionally, the bacterium has a unique cell wall, it localises in the intestinal lamina propria, and it elicits a cellular response that is composed almost entirely of macrophages, with accumulation of bacterial cell-wall remnants in these cells. It has been fairly resistant to cultivation in vitro, and seems to depend on human cell-associated factors for growth, with an in-vitro doubling time that is among the longest known for bacteria.

Genome sequences have provided many insights and clues about bacterial functional capabilities and evolution. By contrast with the medically important bacteria, nearly all bacteria in natural environments and most in the commensal flora have not been cultivated in vitro.[9] The genomes of two other cultivation-resistant human pathogens, *Treponema pallidum*, the agent of syphilis, and *Mycobacterium leprae*, the agent of leprosy, have revealed features that are uncommon among other bacteria with

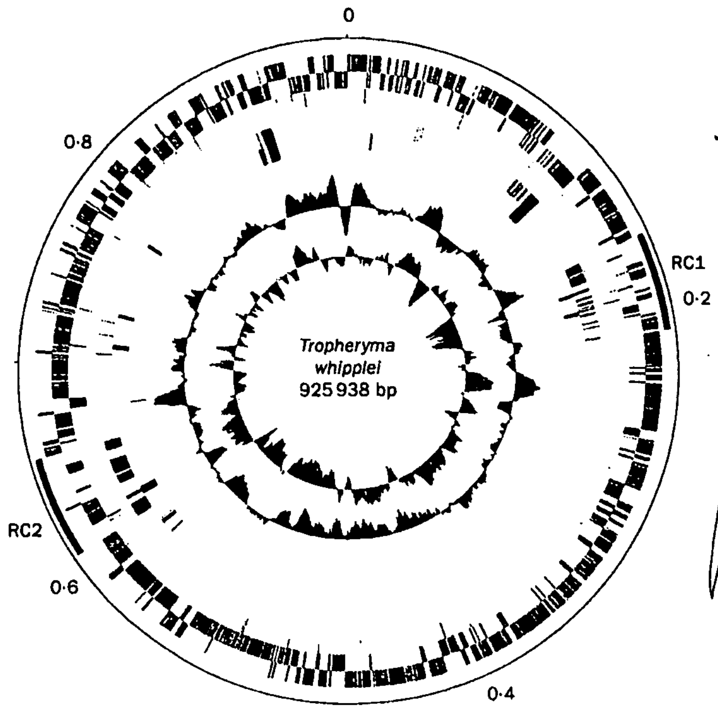

Figure 1: Circular representation of the *T whipplei* chromosome
The outer scale shows the size in Mb. RC refers to repeat clusters (see text). From the outside in, circles 1 and 2 show all genes transcribed clockwise and anticlockwise (see colour code below); circle 3 shows genes for stable RNA (blue); circle 4 shows DNA repeat regions (pale blue=coding repeats, orange=non-coding repeats); circle 5 shows the members of the WISP family (green); circle 6 shows the positions of base-pair sequence variations; circle 7 shows a plot of G+C content (in a 10 Kb window); circle 8 shows a plot of GC skew ([G-C]/[G+C]; in a 10 Kb window). Colour coding for genes: dark blue=pathogenicity or adaptation, black=energy metabolism, red=information transfer, dark green=membranes or surface structures, cyan=degradation of macromolecules, purple=degradation of small molecules, yellow=central or intermediary metabolism, light blue=regulators, orange=conserved hypothetical, pale green=unknown, and brown=pseudogenes.

sequenced genomes. For example, *T pallidum*, with a 1·14 Mb genome, is deficient in genes for catabolic and biosynthetic pathways,[10] and *M leprae* (which contains numerous pseudogenes and evidence of substantial decay and reductive evolution) has maintained almost all biosynthetic pathways while substantially reducing its catabolic and energy-production pathways, leading to its obligate intracellular lifestyle in people.[11]

Until now, genomic characterisation of *T whipplei* has been restricted to the DNA sequences for the rRNA operon and two housekeeping proteins (RpoB and GroEL). The study of the *T whipplei* genome provides an opportunity for new insight into the biology of this enigmatic pathogen and its interaction with human beings, the development of new diagnostic and preventive strategies, as well as the fundamental principles governing the evolution of host-adapted microorganisms. Our aim was, therefore, to generate and analyse the sequence of the *T whipplei* genome.

Methods

We isolated the *T whipplei* strain TW08/27 from the cerebrospinal fluid of a woman in Germany 2 years after presentation with severe weight loss. Her diagnosis of Whipple's disease was based on intestinal histology and 16S rDNA PCR at the time of presentation. The patient had received an initial 2-week course of treatment with penicillin plus streptomycin, followed by 1 year of cotrimoxazole, and had had a therapy-free interval of 1 year before the sample was taken.

Culture was done with a previously described human fibroblast system, initially on MRC-5 cells (CCL-171; American Type Culture Collection, Manassas, VA, USA) and then, in parallel, beginning with the 13th passage, on primary human foreskin fibroblasts (gift from E Mocarski, Stanford University, CA, USA). The initial inoculum consisted of about 10^3–10^4 bacteria in 0·5 mL cerebrospinal fluid. We detected growth after 6 weeks, as assessed by qualitative PCR for *T whipplei*,[12] by quantitative PCR, and by documentation of bacteria by using YO-PRO-1 nucleic acids stain (Molecular Probes, Eugene, OR, USA). 15 culture passages were completed over 17 months, by inoculating 1/4 to 1/5 volume of culture supernatant with bacteria onto fresh fibroblast monolayers. We measured bacterial growth by quantitative competitive PCR with *T whipplei*-specific primers[12] and a synthetic internal standard molecule, and estimated a generation time of 4 days. We identified bacteria in the culture by fluorescence in-situ hybridisation with a *T whipplei*-specific probe and a bacterial broad-range probe,[13] and by broad-range PCR, spanning 1443 bp of 16S rDNA. All bacterial cells hybridised with the *T whipplei* probe, indicating the absence of contaminants. 16S rDNA, amplified with broad range primers after the 15th passage, had two mismatches with the sequence of *T whipplei* (EMBL accession number: X99636) at unpaired positions. We harvested about 10^9–10^{10} bacteria from the culture supernatant of 50, 150 cm² tissue-culture flasks by differential centrifugation (10 min at 950 *g* to remove cell debris, then 20 min at 10 000 *g* to pellet bacteria), and extracted genomic DNA from the bacterial pellet with lysozyme and proteinase K digestion, phenol/chloroform extraction, and isopropanol precipitation.

We sheared chromosomal DNA by sonication, and size fractionated it on agarose gels, before cloning into pUC19 and pMAQ1b vectors (http://www.sanger.ac.uk/Teams/Team53/psub/ref.shtml). End sequences from these clones were generated with dye terminator chemistry on ABI3700 automated sequencers. The final sequence was obtained

Panel 1: General features of the *T whipplei* genome

Size	925 938 bp
G+C content	46·3%
Coding sequences	784*
Coding content	84·4%
Average gene length	998 bp
rRNA	1 (16S-23S-5S)
tRNA	51
Other stable RNA	1

*Of which one is a pseudogene.

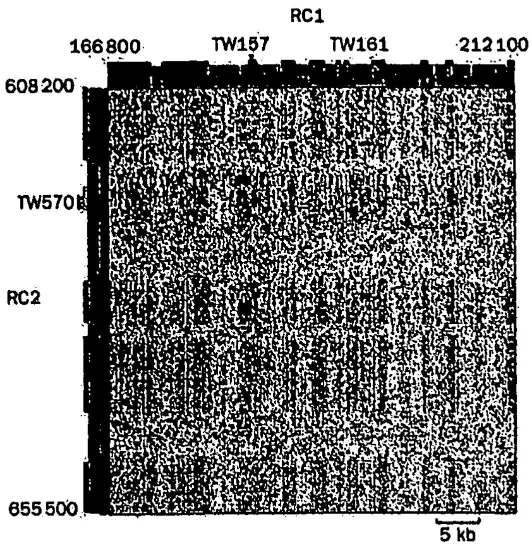

Figure 2: **Dotplot comparison of RC1 and RC2**
The centre of the figure shows the DNA:DNA similarities between RC1 and RC2, with similar sequences represented by dots or lines. The degenerate repetitive structure of the non-coding regions can be seen, along with the copies of these repeats in the WiSP proteins. The conserved N-terminus of the WiSP protein is also visible. The boxes with arrows around the plot show the genes encoded by the region, with the colour code as in figure 1. The non-coding repeat regions are shown by the outermost orange boxes, with the similar repeats in TW157 and TW570 indicated by hatched orange boxes. The positions of variable sequences within TW157 and TW570 are shown by black arrows. Genomic coordinates for the clusters are given in bases.

from 18 156 shotgun sequences (giving 8·9-fold coverage). Of these, 9960 were paired end reads from a pUC19 library with insert sizes of 3·0–3·3 kb, and 7276 were paired end reads from a pMAQ1b library with insert sizes of 5·0–5·5 kb. All identified repeats were bridged by read-pairs or end-sequenced PCR products. The sequence was assembled, finished, and annotated as described previously,[14] using Artemis to collate data and facilitate annotation. Pseudogenes had one or more mutations that would ablate expression; each of the inactivating mutations was subsequently checked against the original sequencing data. We manually checked potentially variable sequences against the original sequencing data. Variations were only considered for subsequent analysis if each of the different bases was clearly present in the sequences from at least two independent shotgun clones. We investigated metabolic pathways with the KEGG database.

Much of the usual chromosomal GC bias[15] was obscured by the anomalous nucleotide biases of the large non-coding regions. However, we were able to place the likely origin of replication near the first base of the *dnaA* gene, the usual point of chromosomal replication initiation in actinomycetes,[16] and this site was therefore chosen as the start of the sequence. SignalP and TMHMM were used to detect proteins likely to be secreted in, or localised to, the cell envelope. Protein clustering was done as previously described.[17] We initially identified and delineated *T whipplei* surface protein (WiSP) family β-strand repeats with Dotter for self-self comparisons, and HMMER (version 2.2g) was used to build a hidden Markov model. We then used this model to search for further copies of the repeat. Potentially phase-variable genes were identified by searching the genome for single base-pair tandem repeats of 9 bp or more (homopolymeric tracts), and tandem repeats of greater than 2 bp with five or more units (heteropolymeric tracts). We further assessed candidates for the likely effect of the tandem repeats on the translation of the associated gene. No significant heteropolymeric tracts were identified.

The sequence and annotation has been included in the GenBank, EMBL, and DDBJ databases with the accession number BX072543. The full annotation and additional references for some of the methods, along with further information and updated annotation are available from http://www.sanger.ac.uk/Projects/T_whippelii.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit the manuscript for publication.

Results

Figure 1 and panel 1 show the general features of the sequence of the *T whipplei* genome, which is 925 938 bp in size and has a G+C content of 46·3%. The genome contains 784 coding sequences, including only one identifiable pseudogene.

More than 5% (46 899 bp) of the chromosome is made up of non-coding repetitive DNA, which has a greatly biased dinucleotide content by comparison with the rest of the genome. This large amount of non-coding repetitive DNA accounts for a fairly low coding density (84·4%) for this organism. The non-coding DNA is located in two clusters, termed repeat cluster 1 (RC1) and repeat cluster 2 (RC2). RC1 is 45 299 bp long with a coding density of just 51·3%, and RC2 is 47 273 bp long with a coding density of 37·8%. RC1 and RC2 are located almost opposite one another on the chromosome map (figures 1 and 2). More than half the coding sequences within RC1 and RC2 encode membrane proteins.

We ascribed a putative function, based on sequence similarities with entries in public databases, to 74% of coding sequences. We designated 84 (11%) coding sequences as conserved hypotheticals, and predicted that most of the remaining 116 (15%) sequences, with no match in the databases, encode cell-envelope or secreted proteins. Of the *T whipplei* predicted proteins with no significant match in the public databases, most (86) are probably exported from the cell cytoplasm and are localised to the cell envelope.

Clustering analysis of all proteins encoded by the genome revealed a prominent family of predicted surface proteins termed WiSP. Alignment and analysis of WiSP sequences revealed a heterogeneous family of proteins with several identifying features (figure 3). Sizes of WiSP proteins range from 103 to 2308 residues. Some of the smaller members could be remnants of larger coding sequences and might not actually be expressed or be functional. It is noteworthy that RC1 and RC2 contain two and one WiSPs, respectively. Ten of the 14 family members have N-terminal secretion signal sequences. In eight cases the signal sequence forms part of an about 300-residue conserved domain (WiSP N-terminal domain; WND), which differs by only one or two aminoacids between different members. The domain is rich in serine and threonine residues and is likely to have a low complexity structure. Five of the 14 contain one or more predicted transmembrane domains near the C-terminus, which could potentially anchor the proteins in the bacterial membrane. TW774 is comprised of a single domain that is similar to the C-terminus of TW113 (94% identity over 120 aminoacids) and TW776 (96% identity over 47 aminoacids).

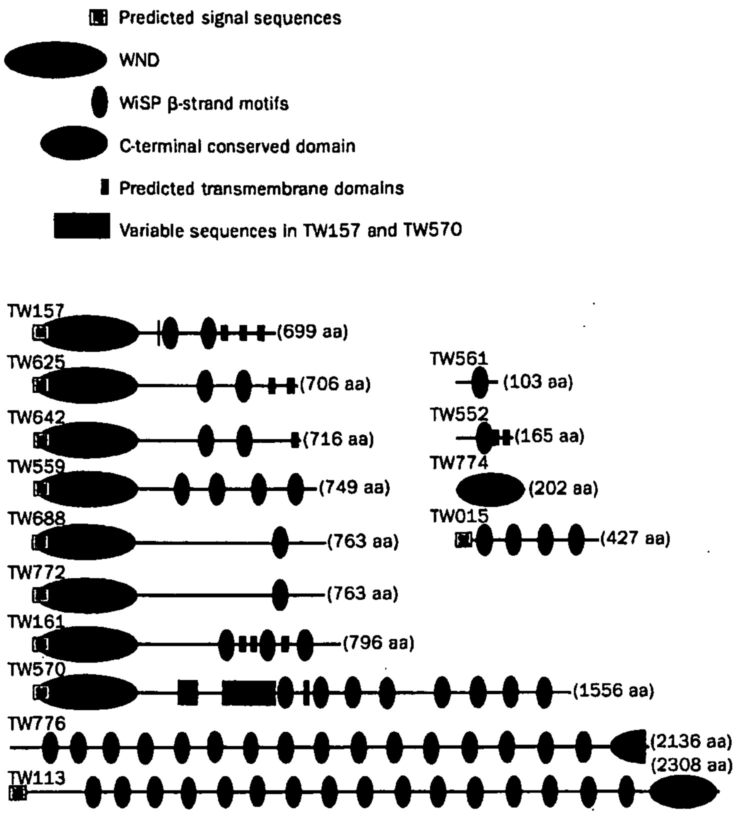

Figure 3: **Architecture of WISP family**

tandem repeat, were located within WiSP coding sequences TW157 or TW570 (three and 44 variable loci, respectively). The effect of most of the variations was to change a single aminoacid. Only two of the variations, both in TW570, resulted in a frameshift mutation. For both TW157 and TW570, the sequence variation was localised. The three single-base variations in TW157 affect two neighbouring codons whereas the 44 loci in TW570 span 338 codons in three clusters (figures 2 and 3). In neither case do the variations affect the WiSP conserved domains. PCR amplification and sequencing of the N-terminal region of TW570 from other passages of the *T whipplei* culture used for sequencing, and from other cultures, also revealed a similar pattern of localised specific variation both within the PCR products and between the products and the final genomic sequence (see webfigure 2 http://image.thelancet.com/extras/03art1087webfigure2.pdf). Different variations predominated at different passages of the same culture. The two genes showing this variation are embedded within each of the repeat clusters described above (figure 2). Although most of the repetitive DNA in these clusters is non-coding, some of the short DNA repeats are identical to sequences located within the two genes, at the regions at which variation arises. For example, a 205 bp sequence, covering the most C-terminal three variants in TW570 is repeated within a non-coding region of RC2 29 kb upstream. The sequences within the non-coding repeat exactly duplicate the minority variants in the TW570 coding sequence (see webfigure 3 http://image.thelancet.com/extras/03art1087webfigure3.pdf).

With the exception of TW774, all WiSPs contain at least one copy of a β-strand motif. An alignment of all of these β-strand motifs is shown in webfigure 1 (http://image.thelancet.com/extras/03art1087webfigure1.pdf). TW113 and TW776 are comprised almost entirely of β-strand motifs, each having 16 copies. WiSP β-strand motif repeats have a median repeat length of 107 residues (mode 89 residues), but only the central portion (about 35 residues) is strongly conserved. The hidden Markov model used to identify these motifs was used to search the SWISS-PROT and TrEMBL protein databases for similar structural sequences. We identified similarities in long proteins of similar organisation—eg, the biofilm-associated protein, Bap, from *Staphylococcus aureus*. Homology searching also revealed similarities to HYR and PKD domains, both Ig-fold structures.

Five genes in *T whipplei* seem to be phase variable—ie, they can be randomly switched on and off by variation in short repeat tracts (panel 2); four are probably integral membrane proteins, and the fifth is TW642, a member of the WiSP group. That this mechanism is active is supported by the fact that three of these coding sequences are frameshifted at the repeat tract (in the off state), and that one of the repeats (downstream of the 5S rRNA) is variable in chromosomal PCRs of *T whipplei* DNA from different patients (data not shown).

Shotgun sequence assembly revealed 48 loci in the genome where alternative sequences existed in the shotgun clones (table); 37 of these were single nucleotide polymorphisms. All but one, a variable length

Panel 2: Potentially phase-variable genes

Coding sequence	Repeat	Location within gene	State	Product
TW143	G (11)	N-terminal	Frameshifted	Integral membrane protein
TW189	G (11)	Promoter?	Intact	Integral membrane protein
TW447	G (11)	N-terminal	Frameshifted	Integral membrane protein
TW587	C (9)	N-terminal	Intact	Integral membrane protein
	C (10)		Non coding	Downstream of 5S rRNA
TW642	G (11)	N-terminal	Frameshifted	WiSP protein

Location (coding sequence, codon)	Sequence variants	Aminoacid variants
TW157, 322	C or A	Ser or Tyr
TW157, 323	A or G	Ser or Gly
TW157, 323	T or C	**Ser**
TW570, 710	ACA or GGG	Thr or Gly
TW570, 705	C or A	Thr or Lys
TW570, 699–701	AACCAA or CTC	ThrThrAsn or ThrSer
TW570, 630	C or A	Arg or Ser
TW570, 620	T or C	Tyr or His
TW570, 617	T or G	Ile or Arg
TW570, 615	T or G	**Ser**
TW570, 615	C or A	Ser or Tyr
TW570, 615	T or G or A	Ser or Ala or Thr
TW570, 613	G or T or C	Gly or Val or Ala
TW570, 587	A or C	Thr or Pro
TW570, 584	A or T	Thr or Ser
TW570, 565	A or T	Thr or Ser
TW570, 557	T or A	Val or Glu
TW570, 548	A or C	Thr or Pro
TW570, 546	TT or TCCCT	Leu or SerLeu
TW570, 544	G or A	Gly or Glu
TW570, 544	G or A	Gly or Arg
TW570, 543	G or A	Arg or Lys
TW570, 542	C or A	Thr or Lys
TW570, 541	T or C	Asp or Asp
TW570, 541	A or G	Asp or Gly
TW570, 541	G or A	Asp or Asn
TW570, 538	C or T	Tyr or Tyr
TW570, 537	A or C	Ile or Ile
TW570, 504–506	CCGTGTTT or GCACAGAG	AlaValPhe or GlyThrGlu
TW570, 500–501	CAAGT or TATGG	ProSer or LeuSTOP
TW570, 497–498	GGTGGT or AAGACC	GlyGly or ArgThr
TW570, 493	C or G	Ala or Gly
TW570, 493	G or A or T	Ala or Thr or Ser
TW570, 492	C or T	Thr or Ile
TW570, 428	G or A	Arg or Gln
TW570, 423	T or A	Ser or Thr
TW570, 421	G or C	Lys or Asn
TW570, 420	C or A	Asp or Glu
TW570, 420	G or A	Asp or Asn
TW570, 398	C or AA	Frameshift
TW570, 393–394	CC or TA	ThrHis or ThrAsn
TW570, 386	CC or CAAA or CATATACCCAATATCTATC	Pro or frameshift or frameshift
TW570, 384	C or T	**Ser**
TW570, 379	G or A	Ala or Thr
TW570, 376	A or T	Thr or Ser
TW570, 374	GG or GGTG	Gly or frameshift
TW570, 372	A or T	Ile or Leu
TW600, 37-57	5, 6, or 7× CTAGAACTA	5, 6 or 7× LeuGluLeu

Synonymous changes in bold.

Aminoacid changes caused by variable sequences

Metabolic reconstruction shows that there are no genes for arginine, tryptophan, and histidine biosynthesis, and there are deficiencies in the ability to synthesise glycine, serine, leucine, and cysteine. Genes for synthesis of biotin, thiazole, and thiamine are absent, as are those for the fumarate reductase and the NADH dehydrogenase complexes. To enable import of essential compounds, *T whipplei* encodes 47 transport proteins, 33 of which are ABC family transporters. Although it is often difficult to predict a specific transport substrate, there is a possible operon of five coding sequences for an aminoacid import system (TW200–204). Oxidative phosphorylation seems to be limited to cytochrome oxidase and ATP synthase. Although glycolysis or gluconeogenesis, pentose phosphate pathway, and pyruvate metabolism seem intact, there is a complete absence of genes for the tricarboxylic acid cycle. The *T whipplei* genome encodes 20 (2·56%) proteins with predicted regulatory function (figure 4).

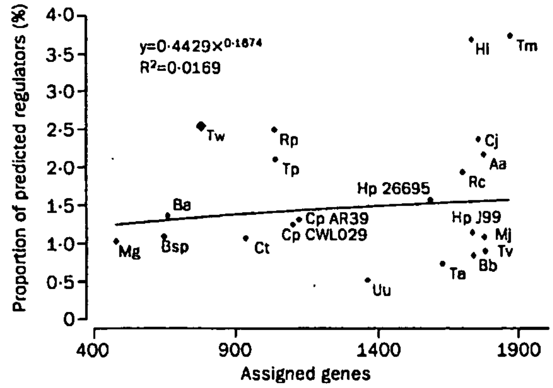

Figure 4: Proportion of coding sequences that encode predicted regulators, as a function of genome size
Data from genomes with less than 2000 coding sequences were obtained from the TIGR Comprehensive Microbial Resource (http://www.tigr.org/tigr-scripts/CMR2/CMRHomepage.spl), using the CMR automated annotation to ensure uniformity. Smaller genomes were selected so as to complement an earlier analysis[37] and provide a more relevant data set with which to compare the *T whipplei* genome. A best fit of the data is shown as a curve described by the equation $y=0{\cdot}4429x^{0{\cdot}1674}$, $R^2=0{\cdot}0169$. Mg=*Mycoplasma genitalium* G37; Bsp=*Buchnera* sp APS; Ba=*Buchnera aphidicola*; Tw=*Tropheryma whipplei* (in orange); Ct=*Chlamydia trachomatis*; Tp=*Treponema pallidum*; Rp=*Rickettsia prowazekii*; Cp=*Chlamydophila pneumoniae*; Uu=*Ureaplasma urealyticum*; Hp=*Helicobacter pylori*; Ta=*Thermoplasma acidophilum*; Rc=*Rickettsia conorii*; Hi=*Haemophilus influenzae*; Bb=*Borrelia burgdorferi*; Cj=*Campylobacter jejuni*; Aa=*Aquifex aeolicus*; Mj=*Methanococcus jannaschii*; Tv=*Thermoplasma volcanium*; Tm=*Thermotoga maritima*.

With respect to DNA repair, the genome only includes coding sequences for a Rec recombination pathway (*recA*, *recF*, *recG*, *recN*, *recO*, *recR*) and the ABC excision excinuclease (*uvrBAC*).

An analysis of coding sequences revealed 14 genes with anomalous dinucleotide content, codon use, or positional base preference (panel 3). Of these, 11 are WiSP proteins.

Panel 3: Genes with pronounced nucleotide anomalies

Coding sequence	Protein	Anomalous dinucleotide content	Anomalous codon usage	Anomalous positional base preference*
TW113	WiSP	+	+	+
TW157	WiSP	+	+	+
TW161	WiSP	+		
TW350	Coiled-coil	+		
TW391	unknown	+		+
TW559	WiSP	+		+
TW570	WiSP			+
TW621	ProAla-rich integral membrane	+		+
TW625	WiSP	+	+	+
TW642	WiSP	+	+	+
TW688	WiSP	+	+	+
TW772	WiSP	+	+	+
TW774	WiSP	+	+	+
TW776	WiSP	+	+	+

*1st, 2nd, and 3rd position G+C content within codons.

Discussion

Our findings indicate that the genome of *T whipplei* has several novel features. First, the bacterium has an unexpectedly small genome, bearing the traits of strictly host-adapted organisms, including pronounced deficiencies in energy metabolism and requirements for external aminoacids. Second, despite the small genome size, the bacterium devotes a large amount of coding capacity to biosynthesis of surface-associated features, suggesting that interaction with its host plays a major part in the organism's lifestyle. Third, the genome reveals mechanisms for generating genetic variability, including phase variation and seemingly directed point mutations. Large regions of non-coding repetitive DNA, unlike anything previously seen in bacterial genome sequences, seem to be a central feature. Most of the variation generated seems to be directed towards changes in cell-surface proteins, indicating that these mechanisms have been developed to evade the host's immune response during the course of chronic disease.

Actinomycete genome sizes range from 1 million bp to 8 million bp[18] and generally have a high G+C content. At just under a million bp, the *T whipplei* genome is the smallest to be completely sequenced and, atypically, has an average G+C content of only 46%. It is noteworthy that the genome contains 784 coding sequences, including only one identifiable pseudogene. This low degree of gene disruption could in part be due to the total absence of mobile genetic elements, such as insertion sequences, within the genome. As bacterial genome size decreases, the proportion of coding sequences dedicated to housekeeping functions increases, as is the case with this bacterium.[19]

Genome analysis indicates that *T whipplei* has many cell-envelope genes typical of actinomycetes. A peptidoglycan biosynthesis gene cluster (TW540–TW551), for example, is largely similar to that seen in other sequenced actinomycete genomes. As with both *Mycobacterium tuberculosis* and *Streptomyces coelicolor*, a class B high-molecular-weight penicillin-binding protein/transpeptidase gene (TW548) is located towards the start of the cluster and the *ftsW* and *ftsQ* cell division genes towards the end. Unlike *M tuberculosis* and *S coelicolor*, the *ftsZ* gene, although present in the *T whipplei* genome, is not linked to this cluster.

The genome contains a gene cluster that seems to direct the biosynthesis and export of extracellular polysaccharide (TW032–042), which could explain a previous observation that the *T whipplei* cell wall includes, in addition to peptidoglycan, an unusual inner layer comprised mainly of polysaccharides.[3] This polysaccharide layer accounts for the positive periodic acid-Schiff (PAS) staining reaction, which is a major diagnostic feature in the histopathology of Whipple's disease. De-novo fatty acid biosynthesis seems to occur via the FasII route—ie, using a series of dissociated enzymes and a small acidic acyl carrier protein (ACP) rather than the multifunctional FasI polypeptide that is common to mycobacteria and corynebacteria. Genes encoding most of the FasII component proteins are clustered together (TW515–519), and there is no evidence for mycolic acid production.

15% of the *T whipplei* predicted proteins, and 74% of proteins with no match in the database, are thought to be exported from the cell cytoplasm and are localised to the cell envelope. The assignment of such a high proportion of *T whipplei*-specific proteins to the cell membrane or wall might reflect the importance to the organism of host interactions.

Our findings indicate that the WiSP proteins encoded in the *T whipplei* genome contain structural sequences similar to those identified in long proteins of similar organisation, some of which have been implicated in pathogenesis and immune evasion. One example is the biofilm-associated protein, Bap, from *Staphylococcus aureus*.[20] The Bap core region represents 52% of the protein and consists of 13 successive nearly identical repeats, each containing 86 aminoacids. The protein projects out from the cell surface, allowing the amino terminus to interact with neighbouring surfaces. We also noted similarities between WiSP β-strand motifs and HYR and PKD domains, both Ig-fold structures; HYR is thought to be involved in cellular adhesion.[21] Taken together, these data suggest that the WiSPs are surface proteins involved in host interaction, at least some of which are anchored in the membrane at the C-terminus, with the Ig-fold β-strand domains being structural elements, projecting the N-terminal domains out from the cell surface.

DNA sequence variation within the *T whipplei* genome is apparent at two levels, both of them associated with surface structures, and therefore potentially important in host interaction or immune evasion. First, *T whipplei* probably uses a process called slipped-strand mispairing to vary the expression of a small number of surface proteins (panel 2). This process, which has been well studied in several other pathogens[22] involves the random variation, during replication, in the length of short tandem repeat sequences, which consequently alters the transcription or translation of the genes containing them, randomly switching protein production on and off. This mechanism leads to phase variation, the random reassortment of this fraction of the complement of surface proteins. Four membrane proteins and one WiSP protein seem to be subject to this mechanism.

The second type of base-pair level variation indicated by our findings is an unusual hypervariation in the coding sequences of two WiSP proteins. Variation was not unexpected, since the culture used to make the shotgun libraries was grown over 17 months through 15 passages. What was surprising was the isolated distribution of the variations. Our results suggest that the large non-coding repeat regions of the genome are involved in generation of the variation in these genes, perhaps by acting as sources of sequence variants that could be copied into the expressed genes by some type of gene conversion event. In view of the difficulty of culturing *T whipplei*, the variations seen could be a consequence of a heterogeneous starting population. However, even if this were the case, the frequency of the variation seen and the fact that the variation seems to be constrained to specific regions and varies though culture passage, strongly imply that it is the result of a specific mechanism. Whatever this mechanism, the result is another level of variation generated in the WiSP proteins, underlining their likely importance in host interaction or immune evasion.

Many other factors indicate that *T whipplei* is a highly host-adapted organism. First, some global features of the genome are reminiscent of the genomes of obligate symbionts and obligate intracellular pathogens, such as members of the genera buchnera and rickettsia, respectively.[23,24] *T whipplei* has a small genome size for an actinomycete. Direct comparison with the complete genome sequences of related organisms such as those of *M tuberculosis* and *Corynebacterium diphtheriae* indicates that the small size is most likely due to gene loss (data not shown). Contraction of genome size due to gene decay

and loss is an important theme in the evolution of host-obligate genomes as the organism adapts to life in a less variable environment.[11,21] Another common feature of chromosomes from host-dependent bacteria is a reduction in G+C content, by comparison with the chromosomes of free-living close relatives.[21] Actinobacteria are characterised as high G+C bacteria, but *T whipplei* has a G+C content of just 46·3%. Reduction in G+C content might be associated with a loss of DNA repair functions,[25] as seems to be the case for *T whipplei*.

The absence of genes required for prototrophic growth is another indicator of reliance by a bacterium on a host for essential compounds.[24] Metabolic reconstruction of *T whipplei* indicates the absence of proteins for several key biochemical steps—eg, aminoacid biosynthesis. Aminoacids that cannot be synthesised must be scavenged from an extracellular source, namely the host. The *T whipplei* genome also suggests deficiencies in cofactor biosynthesis, energy metabolism, and carbohydrate metabolism. These findings suggest that *T whipplei* has become adapted to, or dependent on, its host.

Anomalies in G+C content, dinucleotide frequency, or codon usage could indicate genes or regions that have been acquired by recent horizontal transfer, and may reflect the frequency with which a bacterium has contact with other bacteria.[26] However, none of the 14 genes with nucleotide anomalies in the *T whipplei* genome were adjacent in the genome, and none lay in specific regions with other characteristics suggestive of horizontal transfer. Thus, we conclude that the observed compositional biases are not indicative of horizontal transfer, but reflect other properties, such as biased aminoacid use in the proteins. This lack of evidence for recent horizontal transfer, together with a complete absence of mobile elements in the genome, such as insertion sequences or prophage, is consistent with the notion that the organism resides in a secluded niche, and is not often exposed to foreign bacterial DNA.

One genome feature, however, lends support to a contrary argument and suggests that *T whipplei* senses (and is exposed to) a wider variety of environmental cues. In general, the proportion of genes that encode predicted regulatory factors rises with increasing bacterial genome size.[27] However, there is variability in the proportion of regulator genes among organisms with similar genome size. There is speculation that organisms exposed to greater numbers of environmental conditions require a larger number of regulators.[27] The *T whipplei* genome encodes a high proportion of proteins with predicted regulatory function, suggesting that the bacterium persists in a complex set of environmental niches. Although these could, of course, be alternative sites within the host, *Borrelia burgdorferi* also persists in many host sites and its genome contains a low proportion of predicted regulator genes.

Genes with clear predicted roles in *T whipplei* virulence are infrequent. Two predicted surface proteins TW583 and TW720 are similar to a protein, TadA, required for pilus-mediated tight adherence by the Gram-negative pathogen *Actinobacillus actinomycetemcomitans*.[28] TadA is widely distributed among bacteria and archaea. In *A actinomycetemcomitans*, it is a type IV secretion ATPase that probably energises the secretion and assembly of Flp pili.[29] As in *A actinomycetemcomitans*, the *T whipplei* TadA coding sequences are the first genes in operons of membrane protein encoding genes. The presence of two homologues in the stripped down genome of *T whipplei* suggests they have special importance for this organism.

Acquisition of iron is crucially important for bacterial pathogens in the iron-depleted host environment. For *T whipplei* ferri-siderophore uptake is encoded by a cluster of genes, which include two similar putative lipoprotein receptors. The remaining genes in the cluster encode an ABC transporter with similarity to the enterochelin permease of *Escherichia coli*. Although a homologue of the mycobacterial iron-dependent regulatory protein IdeR is present, no genes with significant homology for known siderophore biosynthesis genes are apparent, suggesting that *T whipplei* might only be able to scavenge xenosiderophores.

Determination of the *T whipplei* genome sequence is a major step forward in the characterisation of this previously poorly understood organism. The information gained from sequencing the *T whipplei* genome, both at the DNA and the protein level, can be used to design diagnostic tests for PCR or serological detection. There is evidence that Whipple's disease is underdiagnosed, so more sensitive tests for *T whipplei* will be a key to uncovering previously unrecognised manifestations, thus improving understanding of the disease.[30]

Contributors

J Parkhill was responsible for overall experimental design and analysis of sequence data. S D Bentley, B Barrell, and M Pallen contributed to sequence analysis and interpretation. C A Yeats did the domain analysis of the WiSP proteins. L Dover and G S Besra contributed to the analysis of the cell envelope components. M A Quail and H T Norbertczak generated libraries for sequencing and contributed to finishing strategies and PCR analysis. L D Murphy and D E Harris were responsible for the sequencing, finishing, and PCR analysis of the genome. A Goble, S Rutter, R Squares, and S Squares contributed to the sequencing and finishing experiments. A von Herbay provided the original clinical specimen from which the sequenced strain was isolated, and reviewed the manuscript; M Maiwald established the sequenced strain in continuous laboratory culture, prepared the DNA used for library construction, and contributed to manuscript preparation; D A Relman contributed to overall strategy for laboratory propagation of the sequenced strain and DNA preparation, assisted with annotation, and had responsibility for manuscript preparation.

Conflict of interest statement

None declared.

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Editorial: The Whipple Bacillus Lives (Ex Vivo)!

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Cultivation of the bacillus associated with Whipple's disease, *Tropheryma whippelii,* has been an elusive goal for many generations of clinicians and microbiologists familiar with this disease. The desire to identify this enigmatic organism has motivated many of these efforts. Many purported successes have later proven erroneous, and many more unsuccessful attempts have never been reported [1]. Cell-free media, animal cells, and animals themselves have all been used, resulting in recovery of a wide range of bacterial species, including members of the *Corynebacterium, Streptococcus, Propionibacterium,* and *Haemophilus* genera. The rough resemblance of *Rhodococcus equi*–, *Mycobacterium paratuberculosis*–, and *Mycobacterium avium* complex–associated diseases in foals, cows, and humans, respectively, to Whipple's disease has been noted; however, pathology closely mimicking that of the latter has never been knowingly and intentionally transferred to another human nor reproduced in a nonhuman host. Despite the unusual cell wall features of this bacillus and its reactivity to the periodic acid–Schiff (PAS) reagent [2], the absence of a specific microbial signature has greatly hindered efforts to evaluate these previous cultivation efforts.

Over the past 10 years, there has been a fundamental change in the approach to microbial identification and taxonomy. This change involves a decreasing reliance on cultivated organisms and their associated phenotypes, such as morphology, antigenicity, and biochemical activities, and an increasing reliance on genotype, that is, nucleic acid sequences [3, 4]. Certain genes, such as that of the small subunit ribosomal RNA (ssu rDNA), accurately reflect the evolutionary history of the entire genome and allow one to determine the relationships of any given organism with all others. By taking advantage of interspersed, highly conserved portions of these genes, one can amplify the intervening, phylogenetically useful sequence directly from infected clinical specimens and identify a previously uncharacterized or novel microbial pathogen [5, 6]. With this "broad-range polymerase chain reaction" (PCR) method, a unique, previously unrecognized bacterial ssu rDNA sequence was amplified from multiple independent Whipple's disease tissues [7, 8]. Phylogenetic analysis of this sequence suggested that the Whipple bacillus is an actinomycete and prompted the proposal of a new taxon, *Tropheryma whippelii* [8]. The *T. whippelii* ssu rDNA sequence now provides the basis for a specific PCR detection assay [8–11]. Armed with this diagnostic tool, Schoedon et al. [12] have tested a clever approach for in vitro Whipple bacillus propagation. The outcome of host infection depends in part upon a complex, local interplay of immune effector cells and cytokines. Pathogens often manipulate these host immune responses to render the local environment more hospitable and to enhance their survival or dissemination [13]; one strategy is to alter the local Th1/Th2 helper T cell profile. Suppression of tumor necrosis factor-α or interferon (IFN)-γ–mediated macrophage activation is a common strategy for microorganisms that choose an intracellular niche. Might one mimic this strategy by treating macrophages with cytokines or hormones that deactivate microbicidal pathways but preserve phagocytosis and thereby promote replication of an organism in a protected intracellular compartment? Interleukin (IL)-4, IL-10, and dexamethasone have been shown to enhance intracellular growth of certain pathogens within human macrophages by suppressing both oxidative and nonoxidative killing mechanisms but without inhibiting bacterial uptake [14, 15]. Schoedon et al. have taken this same approach for cultivating the Whipple bacillus. In this issue of the *Journal,* they provide evidence that *T. whippelii* replicates in the laboratory within human peripheral monocyte–derived macrophages, as well as within a macrophage-like cell line, when these host cells are treated with IL-4 [12].

The potential ramifications of these findings by Schoedon et al. are extensive. Yet, given the long and frustrating history of this disease and organism, one must evaluate this report carefully. In the absence of direct bacterial quantification and any obvious extracellular growth in vitro, how strong is the evidence for microbial replication? The authors relied on two types of data: (1) an increase in both the percentage of cells with visible PAS-positive inclusions and in the number of inclusions per cell and (2) PCR-based detection of *T. whippelii* DNA sequences after a number of cell passages sufficient to eliminate DNA detection after an equivalent inoculum dilution alone, that is, 100- to 1000-fold (in the absence of host cells). In theory, the first type of data might be explained in part by more rapid death of uninfected host cells (leading to an increase in the percentage of PAS-positive cells), although this seems unlikely, and by intracellular redistribution and trafficking of PAS-positive bacterial cell wall. PAS reactivity is difficult to quantitate and is only an indirect marker of bacterial number.

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However, quantitative PCR methods with internal standards would provide a more direct and reliable assessment of bacterial growth in this situation [16].

The exact identity of the organism propagated in this study is another crucial issue. Heart-valve tissue with apparently typical Whipple's disease pathology, from 2 persons, served as the inocula for cultivation [12]. From each tissue sample, a partial bacterial 16S rDNA fragment was amplified using broad-range PCR, from which ~400 bp of sequence was determined. Although these sequences were nearly identical to the corresponding segment of the previously published *T. whippelii* 16S rDNA sequence [8], this amount of primary sequence information is less than desirable. At various stages of tissue cocultivation, a *T. whippelii* PCR-based assay was positive, but the details of assay specificity are not provided. For the purpose of species, and certainly strain, identification, a complete 16S rDNA sequence is probably a minimum requirement; in most cases, additional sequence information from more rapidly evolving genetic loci is necessary. Nonetheless, it appears quite likely that the organism propagated by Schoedon et al. is either *T. whippelii* or a close relative.

What have we learned about the Whipple bacillus and its host from the results of this study? Might we have anticipated these findings from information previously available concerning this organism and its associated disease? First of all, *T. whippelii* appears to have a particular affinity for human macrophages and macrophage-like cells. Perhaps it is no coincidence that this organism elicits a prominent macrophage response during natural infection. Whether or not *T. whippelii* warrants the designation as an "intracellular pathogen," however, is unclear. The cell cocultivation conditions chosen by Schoedon et al. may have biased the outcome toward intracellular survival and growth and may not have provided the bacterium with the extracellular conditions that it encounters and prefers in a susceptible host. The pathology of Whipple's disease is notable for numerous intact extracellular bacilli, with some undergoing binary fission; at the same time, most bacilli within macrophages are at least partially degraded [1]. The same observation was made by Schoedon et al. in vitro, despite the macrophage-inactivating effects of IL-4 [12]. Second, the Whipple bacillus is at least microaerophilic. Third, the requirement for IL-4 treatment of macrophages draws attention to the possibility of a host cellular immune defect and the potential role of a polarized Th2 cytokine profile. Bjerknes and colleagues [17, 18] have suggested that monocytes and macrophages from Whipple's disease patients exhibit deficient microbial degradation capabilities. On the other hand, the effects of IL-4 are pleiotropic and nonspecific; the link between a Whipple's disease host defect and IL-4 may be only indirect. In theory, IFN-γ and its receptor are also possible key players in host susceptibility to this disease. Finally, did phylogeny predict physiology? When the relevant branch of the evolutionary tree is robust, microbial phylogeny sometimes predicts preferred growth conditions; however, because there are few known close relatives of *T. whippelii*, such insights would have been difficult to discern.

In an era of decreasing reliance on cultivated organisms and increasing reliance on rapid and specific molecular or sequence-based methods for microbial characterization, what is the value of propagating an organism such as the Whipple bacillus in the laboratory? With phylogenetically useful sequence alone, microbial identification and evolutionary analysis are possible; predictions can be made regarding metabolic, biochemical, and virulence-associated activities and then further evaluated with consensus PCR and sequencing; growth state might be estimated from quantitative rRNA measurements; and compelling arguments can be developed for a role in disease causation [19]. To the degree that additional genome sequence information may be further revealing, one might "walk" the chromosome of an uncultivated microorganism beginning with the ssu rDNA [20]; it may even be possible to determine a complete genome sequence from such organisms with shotgun cloning methods and powerful sequence assembly algorithms. However, the advantages of a laboratory propagated organism are still substantial.

A viable microorganism, provided with relevant growth conditions, readily reveals its metabolic and virulence capabilities. Disease models and correlates of pathogenicity can be established. Laboratory propagation creates substantial amounts of pure microbial cell mass, with which serologic assays can be developed, monoclonal antibodies elicited, and chromosomal DNA easily prepared. From recombinant chromosomal libraries, virulence-associated genes can be isolated, and the molecular mechanisms of disease causation can be explored. Immunodominant antigens can be cloned and expressed. Diagnosis can then be based on whole cell– or recombinant antigen–based serologic assays or on specific immunochemical and immunofluorescent tissue hybridization. Microbial drug susceptibility can be assessed in vitro. Recombinant antigens may be protective for susceptible hosts.

In theory, all of these advantages can now be realized for *T. whippelii*. In practice, several issues will first need to be addressed. The organism propagated by these authors should be characterized in greater detail. Optimization of growth conditions leading to consistent, high-titer culture yields will be important. One approach might involve cell lines bearing transgenes or genetic defects that render them hypersusceptible to *T. whippelii* growth. Intracellular bacterial degradation needs to be minimized. And, of course, the findings reported herein need to be reproduced by others. But if this work is substantiated, Schoedon and colleagues will have made a key contribution to a fascinating 90-year saga in clinical microbiology. No microorganism is uncultivatible; the real issue is whether we are intelligent enough to understand the sometimes complex and intimate growth requirements of our prokaryotic cousins.

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